DIAGNOSTIC PROCEDURES FOR VIRUS AND RICKETTSIAL DISEASES



First Edition

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PREFACE

THE present volume of *Diagnostic Procedures for Virus and Rickettsial Diseases* is offered in acknowledgment of the increasing demand for a collection of laboratory methods at present applicable to the diagnosis of virus and rickettsial diseases of man. The authors, who constitute the committee, held a panel discussion at the Annual Meeting of the American Public Health Association in October, 1946, and from that the book developed at the instigation of the Co-ordinating Committee of the Laboratory Section

No pretense is made to an exhaustive review of all possible technics nor is it in any manner intended to indicate that the procedures described are standardized to the satisfaction of all workers Rather, the compilations comprise methods found most generally workable by those actively engaged in the field at this stage of progress, at times technics have been omitted that may prove to be more effective when submitted to further trial. Similarly, such physicochemical procedures as ultracentrifugation, filtration, and others are not specifically discussed

Although it was suggested that general chapters might discuss the use of embryonated eggs and tissue culture in virus studies or the principles of neutralization and complement fixation tests the authors decided that even though considerable repetition occurred it was more desirable to incorporate description of those technics in the respective chapters on the individual agents This has resulted in the presentation by different writers of minor modifications in technic and at times in overemphasis of preferences developed through individual experience But these biases are largely justified by the fact that the chapters are written by authorities actively and intensively engaged in the development of working methods for the study of the chosen agent and the related disease Consequently, great effort has not been made, editorially, to obtain uniformity in the detail of presentation or wording

Conceived as a manual for the laboratory worker and student this is not intended to be a handbook of clinical diagnosis or of theoretical

virology. It is a trial flight of a limited nature which the Committee in future revisions will undoubtedly expand and improve as experience indicates.

The book is the handiwork of the authors. The detail of editing has been the responsibility of Mrs. Genevieve S. Reilly, of Ann Arbor; Mrs. Stella B. Barlow has aided considerably in the secretarial duties required; Miss Helen H. Wild has undertaken the tedious duty of confirming and correcting bibliographies. The printers, the Ann Arbor Press, have been extremely co-operative in a period when publishing is a difficult problem. Dr. Joseph E. Smadel has assisted, editorially, in the review of manuscripts on the neurotropic viruses and rickettsiae. My function has been largely that of collator.

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I. INTRODUCTION

THE WIDESPREAD occurrence of sporadic cases and even epidemics of an acute, infectious pulmonary disease which is not caused by bacteria nor influenced by sulfonamide drugs, continues to attract attention The malady, generally known as "primary atypical pneumonia or pneumonitis," is a clinical-pathologic entity of diverse etiology Many descriptions of it have appeared and several bacterial as well as nonbacterial infectious agents are suspected or have been identified as the cause of certain outbreaks [1]

In the course of outbreaks of disease, it has been established that the Agents of Q fever, influenza, lymphocytic choriomeningitis, tularemia, coccidioidomycosis, and even brucellosis may be responsible for certain types of sporadic pneumonitis. However, in recent years the growing importance of psittacosis agents as a cause of sporadic pulmonary disease has been evident without laboratory assistance.[2, 3]

At present, psittacosis infections cannot be differentiated from atypical pneumonia either clinically or at autopsy. The exact relationship of the various psittacosis viral agents in the animal kingdom to sporadic or epidemic pneumonitis is quite confused. At the same time, health workers are keenly aware that failure to recognize a psittacosis infection may have serious consequences to the patient and to his contacts.[4] It is anticipated that the application of dependable laboratory procedures to the systematic examination of human and animal specimens will enhance the present-day knowledge, benefit the patients, and assist the health officer in the ultimate suppression of these infections.

Once an obscure and rare disease thought to be contracted only by persons associated with parrots, psittacosis has recently been diagnosed in human beings who handled other birds and possibly mammals. It has been suggested that cases of human pneumonia have been related to a psittacosis-like virus disease concurrently infecting cats.[5] There is, however, little convincing evidence that the mammalian (feline and murine) psittacosis viruses infect man. Spontaneous psittacosis is distributed widely among the true parrots, lorikeets, cockatoos, and budgerigars (lovebirds or parakeets) living in the bush of Australia[4] and in the neotropical jungle land of South and Central America[6] It is ever present in the commercial breeding establishments of the temperate zone where parakeets (*Melopsittacus undulatus*) are bred and raised[1, 6] Many aviaries have transferred the agent through infected parakeets to pet shops, where it has been further transmitted to canaries, finches,[8] and doves[1]

The fulmars or petrels (*Fulmar glacialis*) of the Faroe Islands and Iceland and the sea gulls along the Atlantic Coast are also infected and have been proved to be sources of human disease[9] Inapparent latent psittacosis is very common in pigeons (*Columba livia*[10]), and the handling of these birds has been responsible for many human infections; persons cleaning pigeon lofts or exposed to dust from them have also contracted the disease There is ample evidence that chickens[11] and Peking ducks[12] may shed the viral agent in their fecal droppings.

Even a casual survey of the available records impresses upon one the high incidence of psittacosis in patients associated with nonpsittacine birds infected with a virus of the psittacosis group. However, it must be emphasized that data continue to focus attention on the importance of infected parakeets and parrots as sources of the human disease. For despite the various repeated attempts to control this source, it remains the prime infectional fount.

Burnet,[13] Smadel[14] and others have advanced the interesting theory that an independently spreading human infection, caused by the psittacosis viral agent, may exist The strain is believed to be moderately contagious for man and is thought to spread from man to man by the aerogenic route. The search for such a humanized strain of virus in this general group has thus far proved fruitless. It is significant that a strain of the psittacosis virus recently isolated from a man who had remained a carrier for 8 years, following infection with an agent of parakeet origin, was found to have preserved its original characteristics. With the methods now available, this strain could only be classified as an avian strain of moderate infectiousness, prolonged residence in the human host had rendered it slightly less pathogenic for ricebirds, parakeets, and pigeons.

It would perhaps be proper to interject here a reminder to physicians and health workers that where psittacosis goes unrecognized, the occupational hazard to them and to their assistants is considerable

Following are the minimal criteria essential for proof that a given case of pneumonitis, in any of its various gradations, is caused by a psittacosis-like viral agent.

1. Isolation of the virus during the acute phase of the disease, the convalescent period, or at autopsy.

2 Demonstration of the appearance of complement-fixing antibodies in low titer during the acute phase of the illness or of a significant rise in titer of the antibodies during the period of recovery

In recording the history of a patient under observation for possible psittacosis infection, it is important to note whether or not he has had contact, however slight, with birds or mammals. As in all heterogenous infection chains, the element of chance here plays a role, and fleeting exposure sometimes escapes notice It should be constantly borne in mind that despite the designation "psittacosis" or the implications of the term "parrot disease," any species of bird, and particularly those enjoyed by fanciers, must be suspected as a source of pneumonitis in-

fection. Psittacosis infection is a constant threat to persons engaged in the breeding, handling, raising, sale, and transport of infected birds There are two possible ways in which the disease may be transmitted to man: through indirect transmission by aerial convection (dust from soiled feathers, from dried droppings, etc.); and through direct transmission of the disease agent from the dead or sick bird, or through man-to-man infection. Reports and observations of man-to-man infections are on the increase

A CLINICAL MANIFESTATIONS

The clinical manifestations of the human disease are remarkably uniform The incubation time, though difficult to establish exactly, usually varies from 7 to 25 days after the initial contact.

The first indications of psittacosis are commonly vague, but the actual onset of the disease is likely to be acute Onset is followed by general malaise and headache and fever, succeeded by chills, severe headache, vomiting, photophobia, and lumbar pains

The pyrexia in psittacosis resembles typhoid fever in that it may be remittent for a few days, after which there is definite lysis During the height of the infection, typical manifestations are restlessness, insomnia, delirium, typhoidal state, nonproductive cough, constipation, and, occasionally, diarrhea with extremely painful abdominal distention and tenderness There is nosebleed in approximately 25 per cent of the cases Rose spots appear occasionally Children usually complain only of tiredness and loss of appetite, however, for a few days they may have a mild fever, accompanied by a "rose spot" rash

The temperature rises rapidly and persists It may fall by lysis during the 2d or 3d week

Compared with the temperature, the pulse is relatively slow, this retardation being one of the outstanding features of the disease In fatal cases the pulse is alternately rapid and feeble, cyanosis and low blood pressure may be marked, collapse at some time during the illness is common The spleen is rarely palpable Phlebitis, or a combined phlebitis and thrombosis, is a frequent complication

In every definite case of psittacosis, the lungs are involved Physical signs develop slowly and are of a migratory character X-ray examinations have indicated that parenchymatous consolidation begins early in the disease and involves sharply defined, irregular parts of the lobes, the lower left lobe frequently shows the primary shadows

Despite extensive respiratory involvement, sputum is scanty, mucoid, and very rarely rusty or blood-tinged To obtain specimens for examination it may be necessary to give an expectorant or use a pharyngeal swab, since the cough, if present, is as a rule nonproductive When secondary bronchial infection takes place, however, the bronchitis produces a copious, purulent sputum

Encephalitic syndromes, such as lethargy or stupor, may appear the second

week. Convalescence is very slow and tedious and not infrequently interrupted by relapse. A leukopenia, although often very slight, is evident in about 25 per cent of the cases. Ambulatory cases may vary in duration from 1 day to a week and are recognizable only in the course of epidemiologic investigations.

Any rapidly fatal pneumonia deserves a most critical investigation for the possible presence of the psittacous viral agent.

B. PATHOLOGIC LESIONS

1. *In man.* In man, the extent of pathologic lung involvement will depend on the stage of the illness at which death occurs. Most of the deaths in the pre-penicillin era took place between the 10th to the 18th day of illness in patients between the ages of 40 to 60.

Changes observed at autopsy are a general septicemia and inflammatory conditions in the lung, pharynx, larynx, and trachea, accompanied by a reaction in the hilar lymph nodes. The spleen may be slightly enlarged, soft and congested in appearance; the kidneys and liver show parenchymatous changes. Microscopic examination usually reveals varying cellular contents in the different alveoli of the focal or lobular areas of pulmonary consolidation. Some are filled with fibrin, red cells, and a few polymorphonuclear leukocytes, whereas in others the congestion and edema are replaced by desquamated and partially degenerated alveolar epithelia and macrophages. In the liver, slightly enlarged and congested, there is parenchymatous degeneration; central or centrolobular focal necroses are the characteristic microscopic lesions. The usual structural changes in the spleen are an acute splenic tumor with relatively small follicles and engorged sinuses filled with phagocytic cells. The bronchioles are usually clear or show the same serocellular exudate found in the alveoli. The mucosa of the large bronchi may be completely desquamated, favoring the secondary suppurative pulmonary complications common when patients die 20 to 30 days after onset.[55, 56]

2. *In birds.* The pathologic lesions in birds (pigeons, ducks, fulmars, finches, and canaries) dead of acute psittacosis are much alike.

Psittacine birds dying during the acute stage of infection show wasted pectoral muscles and, occasionally, an erythematous rash and macules scattered on the body. Internally, there is a serous purulent exudate coating the air sacs of the lungs, a pericardial serofibrinous exudate, and plastic deposits over the capsule of the liver. As a rule, the spleen is enlarged, dark in color, and spotted with fine necroses. A swollen, engorged, and sometimes saffron-colored, heavy liver may be studded with large or small necroses and infarctation. Only rarely will lesions be found in the lungs. The chronic latent stage of avian psittacosis yields essentially negative autopsy findings, with the exception of the slightly or definitely enlarged spleen.[57]

C. SPECIAL CHARACTERISTICS OF THE VIRUS

The bacterium-like psittacosis agent forms characteristic spherical elementary bodies described in the literature as *Microbacterium multi-*

forme, Rickettsia psittaci, Levinthal-Cole-Lillie (L C.L) bodies, or *Miyagawanella.*[18] In smears from infected lungs, meninges, spleen, or peritoneal exudate, the viral agent commonly appears as a minute coccus about 0 3 to 0.4 μ in diameter in clusters and clumps, either intracellularly or extracellularly. Diffusely scattered as single extracellular elements in the smear, it stains purple with Giemsa, red with Macchiavello, deep blue with Castaneda.

Since the essential lesions in psittacosis infection, whether of birds, rodents, or man, invade and ultimately destroy the reticulo-endothelial system, a search for the particulate viral bodies should be directed to the cells of this system.

In the very acute stages of the infection, groups of viral bodies are often present within polymorphonuclear leukocytes. Macchiavello's stain will show the deeply red elementary bodies to be frequently intermingled with irregularly shaped blue bodies ranging in size up to 2 μ or more. These so-called "initial bodies" through progressive multiplication finally become elementary bodies. Smears from the tissues of infected birds contain them in all sizes, from the minutest to the largest. These cocci are present in virulent filtrates even when the material is passed through bacteria-tight filters or collodium membranes having a pore width of 0.6 μ They can be partially sedimented by centrifugation at 15,000 r p m. and are specifically agglutinated by psittacosis antisera. A correlation between the different-sized elementary bodies has been established by studying impression preparations or sections of infected mouse tissue and embryonated chick yolk sac membranes. The study of tissue cultures of these materials (in stained and unstained preparations) at varying intervals after inoculation also aided in establishing this correlation.

The evidence that the minute coccal bodies represent the actual viral agent, and are the elementary bodies, is complete.

1. Developmental cycle. The developmental cycle of psittacosis elementary bodies was first described by Bedson and Bland[19] and Bland and Canti[20] Burnet and Rowntree[21] and Yanamura and Meyer[22] later reported on it. The elementary viral bodies are regarded as the last phase of a complete developmental cycle which takes approximately 48 to 72 hours in the mouse. Briefly, the cycle may be described as follows:

One or more elementary bodies invade a cell and incite (if the cell is healthy) the formation of a single homogeneous mass, which is round

or ovoid in shape. Staining with Giemsa produces a characteristic violet color; with Noble's stain, the mass appears light blue. These so-called "plaques" (Bland and Canti [20]), "inclusion bodies" (Levinthal), or "vesicles" (Rake and Jones [21]) consist of a matrix in which the large initial bodies multiply by fission. At first they divide to elements of comparable size, but as multiplication progresses the elements of division become smaller and smaller until the final elementary body stage is reached again Although immobile in the originally rigid "plaques," the particles become increasingly mobile until finally, in the flexible virus colony, they undergo rapid oscillatory (Brownian) movement. The staining intensity of the matrix decreases in the advanced virus colonies as evidence of its liquefaction

If several virus particles invade bird or mammalian cells, they frequently locate at opposite ends of the cell. Each group is then surrounded by a separate matrix, thus inducing double or triple infectious foci and colonies within one cell. By the time a fully matured virus colony packed with elementary bodies is formed, the host cell dies and autolyzes, releasing myriads of particulate elements which are then capable of invading and repeating the cycle in new cells. That this cycle is repeated is strongly indicated by the fact that the early forms exist even in a virus culture which has attained its maximum infectiousness

A subsidiary process which favors rapid multiplication of elementary bodies may be observed in addition to the regular developmental cycle. Virus colonies with no matrix covering, and resembling vesicles, appear in highly vacuolated host cells with pyknotic nuclei. The "cloaking" of the virus colony with an encapsulating membrane, a phenomenon so striking in the regular cycle, is not always discernible.

II ISOLATION AND IDENTIFICATION OF THE VIRUS

A. PRECAUTIONS

Specimens containing psittacosis virus must be regarded as highly pathogenic and dangerous to handle unless proper precautions are taken. The extreme infectivity of psittacosis agents for man does not recommend the examination of such material in the average public health or clinical laboratory. Well-trained, experienced workers have contracted psittacosis, even when working in specially equipped laboratories under carefully controlled conditions of scientific research.

The scattering of infected material through desiccated fecal droppings attached to the down and feather particles of psittacosis-infected birds was always considered to be especially dangerous; it has been found, in addition, that the manipulations required in preparing antigens for serologic tests are equally dangerous. This is particularly true of the pipetting and centrifuging of suspensions of virus elements, so that a special, isolated laboratory room should be reserved for these practices Scrupulous care must be taken that infective material does not dry out and enter the atmosphere in the form of dust It is also vitally important to prevent dispersal of droplets of the infective material, a result of carelessness in pipetting and other work. All manipulations should be conducted under a hood in which the air is treated with ultraviolet lamps or suitably exhausted. The liberal use of aerosols, such as propylene glycol, is highly recommended Technicians should wear rubber gloves, suitable masks, and a head cover to protect the hair After use, all clothing should be placed in a pillowslip or laundry bag and immersed in a hot, strong soap solution, or it may be steamed The worker should wash his hands and face in hot, soapy water.

A separate insectproof and dustproof room is essential for housing experimentally infected birds and animals; the floor of the room should be treated regularly with dust-fixing oils and the door and window sashes must be sealed with felt material to prevent dust from escaping into halls and adjacent rooms. This room should be restricted to all but the bacteriologist and one well-indoctrinated animal caretaker Whenever feasible, such laboratory work should be entrusted to workers who have passed through a latent or recognized psittacosis infection The animals must be kept in glass jars or stainless steel cages with perforated metal lids, which may be covered with several layers of gauze to prevent escape of dust When work with infected birds is contemplated, specially constructed metal cages, each with a window, built-in feeding cups, and a small aperture closable with gauze, are essential

1. *Immunization of personnel.* Rivers and Schwentker[24] advocate active immunization of personnel with dilutions of live virus subcutaneously administered. In recent years some laboratories[25] have prepared formalin-killed psittacosis antigens, but no definite information is yet available concerning the degree of protection that they afford to man. They produce little or no local and systemic reaction. Persons continuously exposed to inhalation of virus are advised to be injected with these psittacosis antigens and to have their resistance reinforced at 3- to 6-month intervals. Further, their immunologic state should be evaluated by complement fixation tests.

Several laboratory infections have been successfully treated with penicillin An early diagnosis is of value A dosage of 30,000 to 40,000 units every 4 hours is recommended for the first 48 hours of treatment; it should then be dropped to 10,000 units every 4 hours and maintained up to and including the 8th to 10th day.

B. Sources of Material

1. *In man* The material available for examination may be blood, sputum, vomitus, or pleural fluid obtained during illness, or tissues, etc., collected at autopsy. Blood collected during the first week of illness or during a relapse, after having been defibrinated or heparinized, should be inoculated intraperitoneally into mice in 0.5 to 1.0 ml. quantity. Recent observations indicate that the virus can be isolated from the blood much more frequently than previously supposed. Microscopic examination of blood films rarely furnishes useful information at any stage of the illness, although various particles have been seen in them by Coles.[14a] Blood serum specimens should always be collected immediately, when the patient is first seen, and thereafter on the 8th, 16th, and 28th days during the illness and convalescence. These specimens are used to give early and retrospective diagnoses by complement fixation test (see below).

Sputum is the material most likely to give positive results, particularly in the early stages of the disease [26b] Unfortunately, it is often scanty or unobtainable. Every effort should be made to get a specimen, however small in amount, even if expectorants must be used. Proper preservation of this valuable material is very important: it should be promptly frozen in a sealed container and shipped to the laboratory in the frozen state.

Pleural fluid, vomitus, or throat garglings may be inoculated with the hope of positive results. Pleural fluid must be taken aseptically, placed in a sterile tube, and sealed with a rubber stopper The amount of fluid obtainable may vary from 1 to 5 ml.; it may be shipped if frozen and packed in dry ice. Vomitus must be collected in a sterile basin and transferred at once to the laboratory. It will not ship satisfactorily Throat garglings are procured by washing the patient's pharynx with sterile saline. If this material is to be shipped any distance for testing or if the test must be delayed several hours, it should be kept frozen until used

At autopsy, obtain any pneumonic part of the lung, spleen, or liver; if pleural or pericardial effusions are present, they should also be collected. Deposit small pieces of tissues in sterile bottles or dishes and take at once to the testing center, there, they should be kept frozen until used. Tissue sections to be sent any appreciable distance should be wrapped in sterile, gauze pads and placed in waxed envelopes. Put

the envelopes in a tightly sealed container, freeze, pack in dry ice, and ship by air express.

2. *In birds.* Single birds are usually submitted for autopsy in the course of an epidemiologic survey. They may be birds dead of acute psittacosis or those sacrificed in an attempt to find carriers and virus shedders. To prepare a cadaver for shipping, wrap it in cheesecloth previously soaked in a 2 per cent lysol solution; put the bird in a container, freeze it if possible, and ship it, packed in dry ice, by air express. Freezing of the whole bird furnishes the most satisfactory specimens for examination.

If a number of bird flocks are being tested for latent infection, a 10 per cent sample of each flock is sacrificed. This is best accomplished by asphyxiation with coal gas or chloroform. Prepare the cadavers as described above. In order to protect the personnel of shipping companies, never attempt to ship living birds suspected of infection except under special instructions of a health officer.

Blood samples of the larger birds (pigeons, parrots, conures, doves, etc.) are sometimes submitted to the laboratory for use in the complement fixation test

C. PREPARATION OF TISSUE EMULSIONS FOR ANIMAL INOCULATION

The laboratory diagnosis of psittacosis in man or animals is based upon the following procedures: (1) microscopic demonstration of the elementary bodies, (2) the reproduction of the disease in laboratory animals; (3) identification of the strain of virus by cross-protection tests. Until there are enough data to warrant an effort to determine the taxonomic position of the isolated virus agents, the latter procedure must remain the exclusive right of the experimental laboratory.

1. *General directions* All frozen specimens are first slowly defrosted in a refrigerator at 40° F. This usually requires 18 to 24 hours. The specimen is then ready to prepare for inoculations.

Weighed portions of tissue, held in petri dishes, are minced with sterile scissors and then transferred to a sterile mortar or "grinder," where they are ground to a paste with sterile sand, glass particles, or carborundum (size 60). A very convenient grinder is a pyrex test tube 150 by 20 mm in which a narrower but longer and stouter test tube 200 by 10 mm, with a roughened surface, acts as a pestle. There is less risk of contamination with this device than when a mortar is used. Large pieces of organs are minced in a special metal container

which can be hermetically closed and operated on a Waring blendor (Catalogue No. 174246, Central Scientific Company).

After the tissue has been ground thoroughly, the required volume of diluent (plain hormone broth with a pH of 7.4 – 7.6) to make a 10 per cent emulsion is run into the tube, and the whole is thoroughly mixed. If no particular urgency exists, it is best to hold all suspensions in the refrigerator overnight (18 to 24 hours) as this facilitates the diffusion of the virus in the diluent and allows additional sedimentation.

Cultures are made to detect possible bacterial contamination before the organ emulsions are put in the refrigerator. Should the emulsions be grossly contaminated, the bacteria must be partially or completely removed before inoculation.

a. Treatment of contaminated material. Tissue suspensions may be decontaminated in the following four ways. (1) For moderate contamination, light centrifugation (about 5 minutes at 3,000 r p.m.) may suffice (2) Coarse filtration through sand and paper filters may be substituted for (1). (3) Centrifugate or coarse filtrates may be passed through an Elford type gradocol membrane having an average pore size of 1 0 μ. This final filtrate should be injected intraperitoneally in at least 1.0 ml. quantity. (4) Since there is always a definite and sometimes a complete loss of virus through filtration, the use of bacteriostatic agents (antibiotics) is preferred.

On the basis of observations by Morgan and Wiseman[a] and Wiseman *et al*, the following stock solution (TSS solution) is recommended.

Tyrothricin	1: 50,000
Sodium sulfadiazine	50 mg
Streptomycin hydrochloride	250 units/ml
Broth pH 7.6	100 ml

The specimen is diluted with the antibiotic solution to make a 10 or 20 per cent tissue emulsion. After being held in the refrigerator overnight, the bacterial contaminations are, as a rule, killed off. If no growths appear on the plates, the material may be safely injected, even intracerebrally. Very dilute suspensions of the virus may be concentrated by lyophilization from the frozen state.

b Sputum. Before emulsions are prepared cultures are made on blood agar plates; the preparation of films for microscopic examination is optional. To prepare the emulsion suspend the sputum, depending upon its consistency, in 2 to 10 times its volume of sterile hormone

the envelopes in a tightly sealed container, freeze, pack in dry ice, and ship by air express.

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autopsy of mice killed 10 days thereafter; elementary bodies are, as a rule, easily recognizable under the microscope.

b. Grind the normal lungs of clinically healthy mice and make into a 10 per cent suspension; test by intranasal inoculation on mice free from pneumonitis virus. Occasionally, several "blind" passages may be necessary before typical pneumonic consolidations are found upon autopsy.

c. The fatality rate from mouse pneumonitis averaged from 10 to 25 per cent in some colonies of mice observed by Eddie. These animals, at autopsy, showed extensive lung involvement, and elementary bodies were readily demonstrated. Lung suspensions prepared as described above produce fatalities in 3 to 10 days on the first passage, whether inoculated by the intraperitoneal, intracranial, or intranasal route. If all of the mice inoculated by the intraperitoneal route do not die they are sacrificed on the 21st day, and their spleens and livers are used for intranasal reinoculation. Mice so inoculated die in 2 to 10 days, and microscopic examination of the pulmonary consolidations readily establishes the viral agents as belonging to the psittacosis group.

The feline pneumonitis viral agents thus far investigated produce pneumonia in kittens. Baker[4] obtained from cats several strains which when intranasally inoculated produced definite pneumonia, with more than half of the lung substance consolidated. Serial passage increased the virulence, death ensuing 2 to 3 days after inoculation.

To demonstrate production of elementary bodies by the feline pneumonitis virus, remove the pneumonic lungs from the infected cat, weigh, and make up to a 10 per cent suspension in broth. Inoculate the mice intranasally. When impression preparations from the consolidated mouse lungs show typical elementary bodies, further work is obviated. A negative or doubtful microscopic examination indicates further passages, however.

3. *Birds.* A frozen cadaver is placed in the refrigerator overnight for thawing. The bird, which is still wrapped in cloth, is immersed in an antiseptic (2 per cent lysol or cresol 1:10,000) and the ventral feathers are plucked while it is in the fluid. Any exudate about the nasal passages or tail feathers excessively soiled by fecal material should be recorded. Next, remove the liver, noting its size, color, and appearance as to necrosis or exudate. With forceps, carefully raise the gizzard and remove the spleen, which is situated on its dorsal surface. The kidneys and a small part of the lower intestines, with cloacal content,

broth (pH 7.2 – 7.4); emulsify by thoroughly shaking with glass beads in a sterile, carefully stoppered container. Refrigerate the material for 18 to 24 hours at 40° C. for extraction. If large numbers of alpha streptococci and staphylococci are found in the cultures, further extraction may be desirable, sometimes for as long as 3 days.

Centrifuge the extracts for 20 to 30 minutes at 3,000 r.p.m. When the bacterial flora of the sputum sample consists of large numbers of beta streptococci or pneumococci, treat the emulsion with the antibiotics described above. However, prolonged contact of specimens with the drugs must be avoided.

c. Pleural fluid, vomitus, and throat garglings. First determine the extent of bacterial contamination in pleural fluid by culturing on blood plates. Hold in refrigerator at 40° C. until inoculated into mice. Vomitus is also cultured on blood plates to detect the type of contaminant, and when heavily contaminated it is best treated with antibiotics. When a large quantity of vomitus is available, the deposit obtained by prolonged high-speed centrifugation (15,000 r.p.m.) of the filtered material is recommended; use a Berkefeld V filter. Throat garglings are prepared in the same way as sputum.

d. Autopsy material. Smears are made from tissue specimens and stained by the special viral stains. Small pieces of the organs, fixed in bichloride-alcohol or Bouin's solution, are used in histologic studies. From suitable fragments of the tissues a 10 per cent suspension is made, for method see general directions, page 12. The emulsion is cultured first to determine the bacterial content and is then refrigerated for 18 to 24 hours. It is advisable to make at least three mouse passages, using the spleen and liver of the sick or recovered mouse for re-inoculation, before rendering a negative report.

2. *Mammals.* There are only scanty data on the prevalence of pneumonitis in mice, felines (Baker [8]) and possibly hamsters, although its existence has been amply proved a number of times. In fact, it is advisable to suspect all mouse or hamster stocks, and it is imperative to examine them for latent mouse pneumonitis before using in psittacosis studies. The latent existence of mouse pneumonitis may be proved in two ways:

a. Pulmonary consolidation may be provoked by the intranasal instillation of sterile peptone or broth, which is given under ether or barbiturate anesthesia. Lung suspensions so passaged produce lesions with regularity in "clean" mice. Virus can be demonstrated readily at

casional diarrhea. The duration of the illness depends upon the amount, virulence, and toxin-producing ability of the 10 per cent emulsion inoculated.

(2) Gross anatomical findings. Death in 2 to 3 days after inoculation. Little that is abnormal can be seen with the naked eye; the spleen and liver may appear normal in size and architecture. Quite characteristic, and often the only microscopic sign for this stage of the infection, is the bloated duodenum covered with a thin viscous exudate and containing only a little chyme. In some of the animals the surface of the liver and the intestines may be moist and covered with a thin, sticky exudate which generally shows abundant endothelial cells packed with virus particles. Mice will in some instances show enlarged spleens and early liver necroses visible to the naked eye. Microscopically, hemorrhages and necrosis are common in the liver; the cells of both the liver and spleen are packed with viral bodies.

Death in 3 to 15 days after inoculation. The abdominal cavity is filled with a stringy, turbid, fibrinous exudate. The spleen and liver are enlarged and sometimes covered with a thick layer of grayish fibrin, the intestinal loops being glued together by the same exudate. The splenic pulp may protrude. The liver is often studded with fine necroses, and its marginal surface may appear rounded.

Death in 15 to 30 days after inoculation. The abdomen is frequently distended by turbid serous effusions (sometimes blood-tinged) in the peritoneum, pleura, and pericardium. The liver and spleen may be enlarged and friable. Residual necroses may be present in the liver; the kidneys show grayish discoloration and parenchymatous changes. Viral bodies are usually very scanty both in the peritoneal exudate and in the spleen but may occasionally be demonstrated in the pericardial covering.

Mice recovered after an illness of 1 to 2 weeks and sacrificed many weeks after infection, or those sacrificed following the injection of material of low virulence, show few if any marked lesions. In general, the intestines are slightly distended and light. The spleen is conspicuously enlarged and the liver friable and mottled. The kidneys are grayish. Although it is difficult to demonstrate the viral bodies in a tissue smear, passage inoculations have demonstrated them to exist as long as 300 days after the initial infection.

Mice recovered from intraperitoneal infections as a rule possess an infection immunity. When animals injected with material suspected of harboring psittacosis survive to the 30th day, it is imperative to sacrifice them and further passage the spleens and livers into fresh mice. At least three such passages are necessary before a negative report is rendered. There is ample evidence that every known strain of psittacosis, including the S.F strain of Eaton, Beck, and Pearson,[4] under certain conditions produces a latent, chronic, carrier stage in mice. Material from pigeons, chickens, ducks, and mice is fatal only intermittently following intraperitoneal inoculation.

(3) Immunity test. Intraperitoneally inoculated mice should be kept under observation for 20 to 30 days. Sacrifice one or two at the

are then removed aseptically. Note the appearance of the lung, the sex, and the age as to immaturity or maturity. Instructions for preparing emulsions will be found under general directions.

D. SELECTION OF EXPERIMENTAL ANIMALS

1. *Mice.* Unfortunately, the discovery of spontaneous latent psittacosis-like infection in laboratory mice[illegible] has created a constant and vexing complication. Despite this burdensome disadvantage, the laboratory mouse has been the animal of choice for reproducing the disease ever since Krumwiede, McGrath, and Oldenbusch[illegible] in 1930 recognized its high susceptibility to the psittacosis virus

2 *Guinea pigs, monkeys, and others* These animals are liable to a degree of experimental infection. Most react only to certain routes of inoculation, and fatal infections are rare, but such experimental work is nevertheless valuable in that *it permits comparison of the different virus strains*

3. *Birds.* The high rate of latent infections in psittacine birds and pigeons does not suggest them as a routine vehicle for the isolation of virus, but they are useful in comparing virus strains

Ricebirds or Java finches would be an excellent laboratory vehicle because of their extraordinary susceptibility to the viral agents. Unfortunately, their scarcity and exorbitant cost ($2.50 to $5.00 each) are prohibitive to routine laboratory use.

Wild sparrows and house finches, while easy to procure and domesticate, are subject to great flock losses from bird malaria At one time it was believed these birds would aid in laboratory routine, but they have since been abandoned

4. *Chick embryo* When inoculated by the appropriate route into developing hen eggs, all psittacosis viruses grow readily The amniotic cavity, the yolk sac, and the allantoic cavity all yield highly infective virus suspensions.[illegible]

E. ROUTES OF INOCULATION. CLINICAL AND PATHOLOGIC FINDINGS

1. *Mice.*

a. Intraperitoneal inoculation. Administer 0.5 ml. of the prepared 10 per cent sterile emulsion. Virulent material from parrots, parakeets, man, and, *occasionally, canaries,* when injected intraperitoneally in this amount causes death of the mouse in from 3 to 30 days. The average infection lasts 8 to 10 days. A few animals will sometimes recover. A short incubation period following injection of avian material is indicative of fresh infection in the bird. Material from pigeons, chickens, ducks, and mice with pneumonitis, when inoculated intraperitoneally, proves *fatal only irregularly.* (1) *Clinical manifestations.* The clinical disease is not very characteristic in the mouse. Symptoms in the more severe infections consist of ruffled fur, an exudate in the eyes almost sealing them shut, indisposition to move, distended abdomen, and oc-

c. Intracerebral infection. Administer 0.03 to 0.05 ml. of 10 per cent emulsion. Sterile pericardial exudates from pigeons, other birds, and man may be injected safely by this route. Somnolence and paralysis often develop within 24 to 48 hours, followed by death within 3 to 5 days. The feline and murine strains, and in fact the majority of psittacosis viral agents, cause paralysis and death when injected intracerebrally. Emulsions of spleens which are only slightly enlarged, when injected by this route, will produce adequate enrichment of elementary bodies. As a rule, two to three blind passages of a weak virus are required before attempting an intracerebral injection. Excluding the respiratory tract as a route of injection precludes the possibility of accidentally picking up a mouse pneumonitis virus.

Every strain of the psittacosis group of viral agents thus far studied produces an infection in mice by the intraperitoneal route, but not a clinical disease or significant anatomical lesions. A fatal infection can, however, be induced through intracerebral injection.

d. Infection by feeding. According to Fortner and Pfaffenberg,[7] the oral administration of material infected with the psittacosis virus produces a high fatality rate (78 per cent), and in addition to abdominal lesions patchy pneumonic consolidations are common.

2. *Guinea pigs* Most strains of psittacosis are reported to have little effect on guinea pigs when injected intraperitoneally. Strains recently isolated during a pneumonitis outbreak in Louisiana, however, were found to contain an agent which is highly virulent for these animals, irrespective of the inoculation route. The majority of avian and murine psittacosis viral agents bring about pneumonic lesions involving parts of one or two lobes of the lung upon intranasal inoculation of 0.2 to 0.5 ml. of organ suspensions Death follows within 4 to 10 days.

All strains induce fever as high as 40° C, enduring in some pigs for 2 to 4 days, irrespective of route The febrile reaction varies in individual guinea pigs, and may be followed by emaciation The majority of the animals recover, but occasionally one will die

Viral agents passaged through guinea pigs do not diminish in pathogenicity for mice, rice birds, or parrots. Reddened, indurated areas result when pigs are inoculated intradermally[10] The degree and size of the reaction depends on the virus concentration and the amount of toxin present in the inoculum As a rule, the site of intradermal inoculation with the strongest dilution of virus shows a zone of induration of

end of the 10th and 15th day, emulsify the spleens, and inoculate into a fresh group of mice. Should all survive (apart from deaths from the contaminant or from intercurrent infection) all or a part of the group *must be sacrificed, and a pool of the spleens and livers tested in a 2d* or even a 3d passage before the results are reported Alternatively, the surviving mice may be tested by inoculating them (by the same route used in the primary injection) with a known virulent emulsion of the organs of mice which have died of psittacosis. Should any of these mice survive, the indication is that the original inoculum contained an amount of psittacosis virus which was *not sufficient to cause fatal in*fection, although sufficient to produce active immunity. Should all of the mice die, it may be concluded that the original emulsions contained no psittacosis virus.

b. Intranasal infection.[80] Administer 0 03 to 0.05 ml of 10 per cent tissue suspension, which should be instilled under a light anesthesia of ether or barbiturate. (1) Clinical manifestations. Mice subjected to intranasal inoculation of virulent material rapidly develop signs of infection: hunched posture, apathy, and increasingly labored respiration. Death follows within 48 hours to 20 days. With less virulent material, recovery may occur, accompanied by the gradual disappearance of all symptoms The intranasal route should be utilized only as a supplementary procedure

(2) Gross anatomical findings Virus administered intranasally produces widespread consolidations involving either part of the lung or an entire lobe Death may be produced between the 8th to 16th day if virus is given in dilutions of 10^{-4} Discrete foci of pneumonia are manifested as the limiting infective dilutions of virus are approached These areas are gray, almost translucent, 1 to 3 mm in diameter, and lie in apparently normal lungs. Rarely does general congestion of the lungs and fusion of the foci make counting impossible The focal areas appear to enlarge centrifugally and produce terminal lobar consolidation in mice which die specifically, viewed through the microscope, they are sharply demarcated from the surrounding normal lung Structurally, they consist of an interstitial and alveolar accumulation of mononuclear cells and polymorphonuclear leukocytes, wherein the elementary bodies reside Elementary bodies will also appear occasionally in the polymorphonuclear leukocytes in the epithelium of the bronchi or they may be extracellular.

The viral bodies are much *less numerous in smears made from lungs in* which infection has been established for 2 or more weeks, and one may encounter great difficulty in finding them in old lesions

Intranasal inoculation should be utilized only as a supplementary procedure

c. Intracerebral infection. Administer 0 03 to 0 05 ml. of 10 per cent emulsion. Sterile pericardial exudates from pigeons, other birds, and man may be injected safely by this route. Somnolence and paralysis often develop within 24 to 48 hours, followed by death within 3 to 5 days. The feline and murine strains, and in fact the majority of psittacosis viral agents, cause paralysis and death when injected intracerebrally. Emulsions of spleens which are only slightly enlarged, when injected by this route, will produce adequate enrichment of elementary bodies. As a rule, two to three blind passages of a weak virus are required before attempting an intracerebral injection. Excluding the respiratory tract as a route of injection precludes the possibility of accidentally picking up a mouse pneumonitis virus.

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varying size, in the center of which is a white necrotic area. The dermal test in the guinea pig is quite useful in titrating virus dilutions, as it gives one a quick (48 to 72 hours) over-all picture of the strength of the viral agent present in a given preparation.

3. Cotton rats (Sigmodon hispidus)

Cotton rats are successfully infected by the intranasal and intracranial routes Such infections frequently prove fatal. The pneumonic lesions are similar to those in mice and guinea pigs.

4 Syrian hamsters (Cricetus auratus)

To avoid mistake when using hamsters, follow the precautions outlined for mice. The possible contamination of any hamster stock with spontaneous pneumonitis virus necessitates this Intranasal inoculation is most commonly used for the hamster, and the dose is 0 05 ml of a 10 per cent suspension. Death follows in 3 to 5 days Extensive lung involvement will be found at autopsy

5 Pocket gophers (Thomomys bottae bottae)

According to Hoge,[31] this animal is highly susceptible to the virus of psittacosis, and any route of experimental inoculation produces fatal infections Anatomical findings resemble those in the acute infections of mice

Squirrels (Citellus beecheyi). Squirrels can be infected by the intranasal and intracranial routes When injected intracranially they show varying degrees of paralysis

Monkeys (Macacus rhesus or cynomolgus). Intratracheal or intranasal inoculation of the Macacus monkey produces lung lesions resembling those found in human psittacosis Intracerebral injection may induce paralysis, ataxia, and death with meningoencephalitic lesions.[31, 32]

6 *Psittacine birds*

Psittacine birds are not used routinely for diagnostic purposes because of a high incidence of latent disease When used, they are injected by the following methods

a Intraperitoneal inoculation Administer 0 3 to 0.5 ml. of a sterile suspension (10 per cent) for intraperitoneal inoculation. Although this route may be used, it is not the method of choice. If the virus is highly virulent the bird dies of a very acute infection within 24 to 48 hours, and the findings may be confused with that of a sepsis. Clinical and pathologic findings are identical with those in intramuscular injection.

b. Intramuscular injection Intramuscular injection of 0 5 ml. of 10 per cent emulsion produces the best clinical picture and pathologic lesions.

(1) Clinical manifestations. The symptoms are sleepiness, a mortar-like urate mass, and ruffled condition of the feathers, those of the tail often being soiled with greenish fecal material. The sick bird

sits inactively on its perch and is occasionally seized with a characteristic attack of shivering Owing to mucus in the nasal orifice, loss of weight and labored breathing are common. The bird may remain ill for several days and then suddenly die, or it may slowly recover.

(2) Gross anatomical findings Death in 5 to 10 days after inoculation The bird is very emaciated, with a razorblade-like breastbone caused by atrophy of the pectoral muscles; a thick, purulent exudate covers the heart. The liver, enlarged and having marked rounded margins, ranges in color from light red to saffron; approximately 10 per cent of the birds show fresh or partially healed necroses and infarcts. The spleen may vary in size from 4 mm. to 10 mm ; it is congested and soft When the lungs are involved, a few consolidated patches of pneumonia appear in the lower parts of one or both lobes

Inclusion bodies can be seen readily in impression smears of exudates or organs Mouse inoculation will demonstrate the presence of the virus in every organ, as well as in the blood Tissue cultures, whether aerobic or anaerobic, are usually sterile Culturing is essential in bird infection due to the fact that birds are highly susceptible to and carriers of Salmonellosis, the pathologic lesions of which are indistinguishable from psittacosis

The characteristic proliferation in and destruction of the reticulo-endothelial system in the liver and spleen are apparent upon histologic examination of the organ structures The spleen often shows innumerable monocytes The tubular epithelial cells of the kidney, the epithelial cells of the glomerular capsule, and the densely infiltrated interstitial spaces between the tubules are the sites where psittacosis inclusion bodies grow

c. *Intranasal infection* Although this mode of infection is rarely chosen, it may be valuable in the study of pulmonary psittacosis lesions in birds. Lobular consolidation of varying degrees accompanies the lesions of septicemia such as in birds injected by the intraperitoneal and intramuscular routes

Birds dying after 30 days or those that apparently recover may be suspected of latent infection Autopsy findings in latent infection are negative, except for the spleen, which may be slightly or definitely enlarged The presence of virus in the spleen can be proved by mouse inoculation It should be stressed that not every enlarged spleen will contain the virus, nor will every apparently normal spleen fail to harbor it This fact clearly indicates that the liver, kidneys, and intestines, as well as the spleen, must be tested in every latent infection Though microscopic examination of smears made from the organs of latent carriers rarely discloses typical inclusion bodies, even over a long period of examination, there are certain small, intracellular, acidophilic, disc-like bodies that may be of some diagnostic significance

7. *Pigeons.* It will be remembered that Bedson and Western[43] were unable to infect pigeons It has since been generally accepted that individual birds of this species may be resistant.

a. Intramuscular and intraperitoneal injection. The dose is 0.5 to 1.0 ml. of a 10 per cent emulsion.

Most pigeons appear to stay well, but it is known that the virus may lie dormant in the spleen or kidney for many months. The occasional pigeon fatalities occur from the 5th to the 40th day.

The clinical picture is essentially the same as in the psittacine birds; ruffled feathers, loose droppings, and marked loss of weight.

The pathologic picture is also closely related to that of the psittacine birds. The most common findings, however, are an enlarged spleen and a thick, gray, pericardial exudate.

b. Intracerebral inoculation. Use 0.1 and sometimes 0.2 ml of the customary 10 per cent suspension of virus to be tested.

The clinical picture of the intracerebrally infected pigeon includes paralysis and refusal of food. The bird dies on the 5th to the 11th day with meningitis.

Pathologically, the picture at autopsy grossly is that of a normal-appearing bird; the brain and meninges may be injected very slightly or show marked congestion, with considerable moisture. Smears made from the spinal fluid or meninges contain numerous elementary bodies, and cultures of the same material are bacteria-free.

c. Intranasal inoculation. Pigeons are quite refractory to this route of infection.

Doves. Doves react the same as pigeons.

8. *Chickens.*

Young chickens may be infected with certain viral agents by the following routes.

a. Intramuscular or intraperitoneal inoculation. Give 0.5 to 1.0 ml. of a 10 per cent emulsion. If the strain is strong in toxin, the chicken dies within 6 to 15 days. It has been found that adult chickens can be inoculated repeatedly with 2 to 3 ml of a 10 to 20 per cent suspension with little effect other than an occasional loss of weight. When an injected chicken is about to die, the clinical picture is that of a droopy, ruffled, dull fowl. Pathologic findings are few. occasionally the spleen is enlarged; the liver may be engorged and the margins noticeably rounded.

b. Intracerebral inoculation. The dose is 0.1 to 0.2 ml. of a 10 per cent emulsion. The rare infection that occurs has an incubation period of 5 to 10 days and presents a clinical picture typical of a meningitic or paralyzed bird. Although intracerebrally inoculated chickens may die within a day, they often linger 5 to 10 days or, like the pigeon, entirely recover. Pathologically, the organs and viscera appear normal in color and size. The brain is moist, with an injected dura.

9. *Embryonated hen's egg.*

a. Chorioallantoic membrane.[24] Inoculate 0.05 ml. of the emulsion to be tested directly into the chorioallantoic membrane of a 10- to 12-day embryo. Distinct poxlike lesions appear on the membrane in from 3 to 6 days, followed by death. This method is used to isolate virus from tissues experimentally and to study the life cycle of the viral agent.

b. Yolk sac inoculation. This procedure is utilized extensively by all virus workers, since it is the ideal method of enriching virus growth and of producing large quantities of infectious material with little contamination. It is simple and direct.

The egg is first candled, and the air sac and yolk sac marked. Using a syringe with a 20-gauge needle 1½ inches long, inject 0.5 ml. of a broth suspension directly into the yolk sac of a 6- to 7-day fertile egg. Cover the needle puncture with a paraffin-beeswax mixture and incubate the egg at 37° C. Candle the eggs each day. When the embryo is becoming enfeebled, it should be watched closely by candling several times a day; it usually dies in 3 to 8 days, depending upon the dilution of inoculum used. After the shell of the egg has been carefully disinfected, it is opened with a sterile forceps and scissors. Open the top of the egg, expose the yolk sac, and aspirate the yolk material with a sterile, large-gauge needle. Carefully lift the yolk sac from the egg with sterile forceps and place it in a sterile petri dish. It should be washed with care in sterile saline until free of yolk material. Impression smears are made and stained to isolate the elementary bodies, because no microscopic lesions have been noted after yolk sac inoculation.

For preparing antigen and titrating virus use a 10-fold dilution of yolk sac membrane in broth, as this amount will cause death of the embryo in 3 days. Dilutions inducing death of the embryo in about 6 days should be used for maximal elementary body production.

c. Allantoic inoculation. This is the best method for obtaining

concentrated elementary bodies. The egg is candled, and the air sac and allantoic sac are marked. A 9-day embryo is preferred. Disinfect the shell and inject 0.25 ml. of yolk sac membrane suspension or 0 25 ml. of allantoic fluid directly into the allantoic sac. The dilution most suitable for the inoculum is that which will kill the embryo in 4 days. Each egg yields from 5 to 10 ml. of clear allantoic fluid. Chilling the eggs overnight in the refrigerator before harvesting increases the yield.

The resultant preparation consists almost entirely of elementary bodies, with very little extraneous material.

d. Amniotic cavity inoculation. This is not a popular method, inasmuch as it has no advantages over the others described. 0.25 ml. of virus dilution is inoculated in the amniotic cavity of a 12-day embryo. Embryos inoculated with a dilution which causes death in 3 to 4 days give the most suitable material for study and for further inoculations.

10. Obstacles to be overcome.

a In mice. The deceptive presence of pneumonitis virus in the experimental mouse used in studies on the cause of human pneumonias easily leads to false conclusions This cannot be overemphasized It is particularly a pitfall when the intranasal route alone is used to test specimens of sputum, blood, and organs Therefore, any laboratory contemplating a diagnostic service for psittacosis or psittacosis-like infections must secure or breed mice which have been proved free from mouse pneumonitis by repeated testing of adequate samples (50 to 100 of mice) every several months

In addition to this, there is in the course of passage experiments a great risk of activating some other infection latent in the mouse. Latent ectromelia and the bacterial infections (Salmonella) are a threat The staining reactions of the ectromelia viral agent differ markedly from that of psittacosis In fact, the cell inclusions characteristic for ectromelia cannot be demonstrated with the Macchiavello's stain, since the virus bodies are acidophilic, as contrasted with the basophilic staining characteristics of the psittacosis bodies

b In birds Salmonella infection is common in birds Acute psittacosis and Salmonella infection can be almost identical, anatomically Routine culturing on blood and enteric media is essential

c. Other viruses to be ruled out Most likely to cause confusion in human infections is the virus of lymphogranuloma venereum The psittacosis patient's serum often reacts with both the psittacosis and Lygranum antigens, in some instances to the same titer Lymphogranuloma virus has been isolated from human sputum According to Rake,[20] lymphogranuloma has been the causative agent of viral pneumonias It has also been found in autopsy material inoculated into mice by the intranasal and intracerebral routes

The lymphogranuloma viral agent differs from the psittacosis agent in that it is sensitive to the sulfa drugs and rarely if ever infects mice by the intraperitoneal route.

Attention is also called to the elementary producing bodies in trachoma and inclusion blennorrhea (eye infection) q v

F. PRESERVATION OF VIRUS

Field specimens under test for psittacosis may be held in lusteroid tubes, glass vials, or other tightly sealed containers. Freeze specimens at 70° C. and keep frozen until used for animal inoculation Such material may be stored safely for one week to several months. It is not advisable to keep it any longer than several months, as a gradual loss of infectivity takes place, especially if the gross material is not sterile.

If fairly clean, the material may be held for several days in the ordinary refrigerator (temperature 0 to 4° C.) without noteworthy loss in potency. Organs of mice or birds heavily infected with virus may be kept frozen for at least 4 years without noticeable effect. Strains of the psittacosis virus are best preserved for long periods of time when desiccated from the frozen state (lyophilization). Store them *in vacuo* in hermetically sealed tubes, in the refrigerator. Experience has shown that under such conditions the agent remains active after 4 to 5 years

Glycerol inactivates the virus

G STAINING OF ELEMENTARY BODIES

Impression smears should be made to confirm the presence of virus in all animal and egg material.

Smears and impression preparations of the exudates or organ surfaces are made on thoroughly clean grease-free slides. Although the prepared slides may be stained by any of the listed methods, the Castaneda and Macchiavello stains are most satisfactory for routine examinations

The staining reactions and morphologic appearance of the viral bodies *are so characteristic that identification in smears from the in*fected tissue of the bird, mouse, or mammal is usually easy In spontaneous fatal infections of birds and in acute deaths of experimentally infected mice and birds, the virus colonies may be seen in all the diffent stages of development. Some experience is no doubt necessary in recognizing and differentiating the viral bodies from other cytoplasmic granules situated in the exudates and tissue and from the fat globules in the *yolk sac preparation, but with the aid of the* Macchiavello's stain, the bright red elementary bodies should be readily identified even by the beginner.

1. *Staining methods.*

a. Castaneda's stain.

(1) Bedson and Bland's modification. Prepare the staining solution as follows:

Phosphate buffer solution according to Wilcox and Logan, pH 7 0	95 ml.
Formalin (neutral accurately 40 per cent)	5 ml.
Borrel blue (= Azure II; for preparation see Langeron[86])	10 ml

Stain film for 2 minutes, rinse with tap water, and counterstain for a few seconds with 1.0 per cent aqueous safranine; dry by blotting

(2) Lepine modification Dissolve Azure II 1 gm in 0 5 per cent phenol water. This stock solution keeps well for many months. To prepare the staining solution add to 10 ml. of distilled water:

5 drops of 1 per cent potassium carbonate
10 drops of Azure II solution.
10 drops of neutral formalin

Flood the film with the stain and heat gently until steaming. Stain with hot solution for 5 to 10 minutes; wash with tap water; counterstain with safranine (1 in 2,000 in distilled water) for 5 to 10 seconds; rinse with tap water; dry by blotting.

b. Macchiavello's stain

Prepare the following stock solutions:

Basic fuchsin	0 25 gm in 100 ml of double distilled water
Citric acid	1 gm in 200 ml of double distilled water
Methylene blue	1 gm in 100 ml of double distilled water

After drying in air, the smear or the impression preparation is fixed by heat. The basic fuchsin solution, first passed through filter paper in a small funnel, is dropped onto the film and allowed to remain for 5 minutes The fuchsin is then drained off, and the slide quickly dipped for a few seconds into the citric acid solution, which is best held in a Coplin jar. Wash the slide thoroughly with tap water and stain with 1 per cent methylene blue for 20 to 30 seconds, wash again in tap water, and dry by blotting Long exposure to citric acid will decolorize the elementary bodies of the psittacosis agent, and they will all stain blue. In a properly prepared slide, most viral bodies will be stained red.

c. Giemsa's stain.

This stain gives excellent permanent preparations, provided a reliable brand of stain (National Aniline and Chemical Company, Incorporated, New York) is used.

(1) Giemsa's stock-staining solution. Dissolve 0 5 gm. of powder in 33 ml of highest purity gylcerol at 55° to 60° C. for 1½ to 2 hours. To this add 33 ml of absolute methyl alcohol, acetone-free. Mix thoroughly and allow to sediment overnight in a desiccator to prevent absorption of moisture. Pour off into small bottles and stopper tightly. In preparing the stain for use, make up dilutions with absolutely neutral distilled water (orange with neutral red or purple with hematoxylin) or with buffered water A trace of lithium carbonate (1 per cent) will usually suffice to give the desired reaction to stock distilled water

d. Buffer water solutions according to Wilcox and Logan's method

(1) Prepare M/15 $NaH_2PO_4 \cdot H_2O$ by dissolving 9.2 gm of the anhydrous salt in 1 liter of distilled water, and

(2) M/15 KH_2PO_4 using 9 07 gm liter

To make buffered water with pH 7.2, mix 72 0 ml of (1) and 28 0 of (2) with 900 ml of distilled water

Although the rapid staining method ordinarily used for blood smears may yield satisfactory preparations, the slow method is recommended, since the Giemsa's stain is largely used in studying the finer aggregates of virus colonies within the cells Following is a description of the slow method.

The fixed thin films are placed in a Coplin jar in a dilute Giemsa's staining solution (1 drop to 5 ml of neutral buffered water) Hold the jar in an incubator at 37° C overnight (24 hours) After removing the slides from the stain, rinse thoroughly with distilled water, then dry between blotting papers To differentiate the stains, the thin film is dipped rapidly in absolute alcohol (in case the preparation is overstained, use 95 per cent alcohol), it is then washed in distilled water, dried, and examined with an oil immersion lens. Permanent preparations may be made by carefully removing the cedar oil Although unmounted dry specimens are more durable, the preparations may be mounted by applying a cover slip and using cedar oil or neutral balsam

2. *Staining of sections* The best solutions for staining sections are Giemsa, Heidenhain's classical iron hematoxylin, and Noble's stain,

applied to material fixed in Zenker's, Bouin's, or Schaudinn's solutions. The sections fixed in corrosive of sublimate must be treated as follows:

(a) Remove paraffin from section with xylene, 5 minutes.
(b) Remove xylene with absolute alcohol and 95 per cent alcohol
(c) Transfer to 70 per cent alcohol tinged with Lugol's solution for 15 to 30 minutes, until light brown in color
(d) Place in 95 per cent alcohol to remove iodine
(e) Pass through 70 and 50 per cent alcohol to distilled water.
(f) Place in 0 5 per cent aqueous solution of sodium hyposulfite for 5 to 10 minutes
(g) Wash in running water.
(h) Transfer to distilled water, stain, differentiate, dehydrate, and mount according to the staining method chosen

a. Polychromatic Giemsa stain. (Wolbach's modification).

Beautiful sections are obtained after fixation of the tissues with corrosive sublimate. Transfer the sections from distilled water to the following solutions:

Distilled water	.100 ml
Giemsa solution	60 drops
Methyl alcohol	.10 ml
0 5 per cent aqueous sodium carbonate	2 drops

Stain for 24 hours, renew the staining solution twice Rinse in distilled water and differentiate as follows:

(1) Fifteen per cent colophonium in acetone for a few seconds.
(2) Transfer to the same reagent for a few seconds
(3) Place in a mixture of acetone 70 parts and xylene 30 parts
(4) Transfer to a mixture of equal parts of acetone and xylene.
(5) Clear in xylene and mount in neutral cedar oil, never in balsam

Control the progress of differentiation under the microscope.

A modification of Schaudinn's solution consisting of equal parts of a saturated solution of mercury bichloride ($HgCl_2$) and acetone, with 5 per cent acetic acid, is particularly useful when using the Noble's stain.

b. Noble's stain.

Prepare sections from carefully embedded paraffin blocks and stain as follows:

(1) Steam section flooded with 1 per cent basic fuchsin solution over an alcohol lamp for 45 seconds.

(2) Decolorize and differentiate in 0.5 per cent citric acid solution for 1½ minutes.
(3) Wash in distilled water.
(4) Counterstain with 2 per cent aqueous orange G solution for 5 minutes.
(5) Rinse lightly and counterstain with 1 per cent aqueous methylene green solution for 5 minutes.
(6) Differentiate rapidly in 95 per cent alcohol.
(7) Pass rapidly through absolute alcohol into xylol.
(8) Mount in balsam or euparal

Properly stained sections will show deep red elementary bodies, green cell nuclei, orange-green cytoplasm, and red to pink erythrocytes.

H. Immunologic and Serologic Identification of Virus

A viral agent whose tinctorial characteristics and developmental cycle are similar in smears prepared from the infected mouse, bird, or chick embryo yolk sac may be readily confirmed as belonging to the psittacosis group by the complement fixation test.

Allantoic fluid or yolk sac material infected with the suspected agent is sterilized by heat or with 0.1 per cent formalin and serves as antigen in the complement fixation test with a known hyperimmune animal or human convalescent serum Psittacosis agents thus far isolated from man, birds, and mammals all have closely related antigenic components Thus, a serum prepared in guinea pigs from a parakeet psittacosis virus with a complement fixation titer of 1.1,280 for the homologous agent reacts in the same concentration, or even higher when, for example, an antigen prepared with the agent of feline pneumonitis or lymphogranuloma venereum is used.

The complement fixation test has been of limited assistance in differentiating the individual members of the lymphogranuloma-psittacosis group The basic differences in source between the members of the group have been recognized, however, and the ever-increasing impact of the concept of species and type differentiation of infective agents has led to a search for procedures to identify and subclassify the various agents *

* Serum of pigeons, mice, and man infected with the psittacosis agent may strongly react with psittacosis antigens but give no cross-reactions with lymphogranuloma antigen (Lygranum)[304] Vice versa, the serum from mice infected with lymphogranuloma reacts positively with Lygranum antigen but fails to fix complement in the presence of psittacosis antigens.[304]

[illegible]

[illegible]

[illegible]

a. [illegible]

[illegible]

[illegible]

Stain for 24 hours, remove the staining solution [illegible] distilled water and differentiate as follows:

(1) Fifteen per cent of [illegible] in acetone for a few seconds.
(2) Transfer to the same reagent for a few seconds.
(3) Place in a mixture of acetone [illegible] parts and xylene [illegible] parts.
(4) Transfer to a mixture of equal parts of acetone and xylene.
(5) Clear in xylene and mount in neutral [illegible]

Control the progress of differentiation under the microscope.

A modification of Schaudinn's solution consisting of equal [illegible] a saturated solution of mercury bichloride ($HgCl_2$) and [illegible] 5 per cent acetic acid, is particularly useful when using the [illegible] stain.

b. Noble's stain.

Prepare sections from carefully embedded paraffin blocks [illegible] as follows:

(1) Steam section flooded with 1 per cent basic fuchsin [illegible] over an alcohol lamp for 45 seconds.

(2) Decolorize and differentiate in 0.5 per cent citric acid solution for 1½ minutes.
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* Serum of pigeons, mice, and man infected with the psittacosis agent may strongly react with psittacosis antigens but give no cross-reactions with lymphogranuloma antigen (Lygranum).[40b] Vice versa, the serum from mice infected with lymphogranuloma reacts positively with Lygranum antigen but fails to fix complement in the presence of psittacosis antigens.[40c]

Early attempts to differentiate members of the lymphogranuloma-psittacosis group by comparative pathogenicity tests on birds resulted in contradictory reports on the same strains from different laboratories. Strains claimed by some to produce no latent infections in mice were found by others to persist in the liver and spleen of the animals for over 85 days. Investigations on the tissue tropism of mouse pneumonitis viral agents were made meaningless by the discovery of new strains. The test for susceptibility to sulfonamides also lost its value when it was recognized by Wiseman, Meikeljohn, Lackman, Wagner, and Beveridge[27] that two human psittacosis strains were affected by the drugs, and deBurgh, Jackson, and Williams[28] encountered mouse pneumonitis strains which resisted the action of the drug Cross-active immunity tests have tended to expose certain differences between the strains when only a few strains were used in the tests Smadel, Wall, and Gregg[10] state, however, that mice resistant to the psittacosis virus 6BC are also resistant to the maximum amount of pigeon virus. Moreover, it is becoming increasingly apparent that strains of low pathogenicity do not always protect mice against intracerebral challenge infections and are therefore immunogenically impotent Similar tests with homologous and heterologous mouse pneumonitis strains by Kempf, Wheeler, and Nungester[28] have not proved very dependable

The serum neutralization test, which has proved invaluable in identifying the members of other groups of infective agents, was not used until recently, when Hilleman[37] produced protective sera of relatively high titer and sharp specificity by the intraperitoneal inoculation of chickens Serum cross-neutralization tests conclusively demonstrated that an antiserum prepared against the meningopneumonitis virus of Francis neutralized only the homologous strain and a pigeon psittacosis virus. A mouse pneumonitis virus neutralized 2 mouse viruses, but had no effect on a human or parakeet psittacosis agent The lymphogranuloma venereum serum acted specifically on the homologous strain

Although the procedures recommended by Hilleman are relatively simple, they do require the skill of experienced workers in research laboratories Until the merits of the method have been properly evaluated, it is recommended that strains of psittacosis viral agents be referred to laboratories properly equipped to conduct the tests

Rake and Jones[38] and Hamre and Rake[39] have demonstrated that the endotoxins in yolk sac suspensions from moribund chick embryos infected with the agents of the psittacosis-lymphogranuloma group are

specifically neutralized only by homologous antiserums. To date, this method has been employed in the study of a limited series of psittacosis viral agents. The methods of immunization and the technic of the test are described by Rake in the section on lymphogranuloma venereum.

III. SEROLOGIC PROCEDURES FOR DIAGNOSIS OF DISEASE

A. COMPLEMENT FIXATION TEST

It was demonstrated by Bedson [40] and later by Meyer *et al.*[41] that serums from mammals, psittacine birds, pigeons and doves, infected with the psittacosis viral agent fix complement in the presence of their homologous antigens and agglutinate the elementary bodies

The test is particularly useful in surveying shipments of imported psittacine birds and flocks of pigeons Since complement-fixing properties are present in the majority of these birds when in a latent state of infection, it is the best tool for quickly surveying possible sources of infection.

1 *Blood.*

a Human blood. Blood should be collected on the 1st day that the patient is seen and thereafter on the 8th, 16th, and 28th days during the infection and convalescence, the blood sample (10 to 15 ml.) must be collected aseptically from the vein and allowed to clot. If it is to be shipped for testing, the serum must be removed aseptically from the clot and sent by air mail

b Bird blood This test has proved valuable in making epidemiologic surveys among birds suspected of infection and for testing individual birds. Blood samples of the larger birds such as pigeons, parrots, cockatoos, conures, macaws, doves, etc, can be obtained easily with a 2 to 5 ml syringe and a 24-gauge needle The blood is usually taken from the wing vein A few feathers on the under surface of the wing are first plucked to expose the vein, and the skin surface is prepared with carbolized 70 per cent alcohol. Insert the needle carefully into the vein and quickly withdraw 2 ml of blood After removing the needle, compress the vein with a pad of sterile cotton to halt further bleeding Amazon parrots, cockatoos, macaws, and birds of like size may be given a light anesthesia when they are to be bled This enables the assistant to hold the bird more easily while the operator works [41]

If the blood is put into tubes which have been first prepared with a very thin coating of sterile vaseline, evenly applied, the yield of clear serum will be greater. For shipping, carefully withdraw the serum into the prepared tube, cork tightly, label, and send by air express.

2. *Antigen.*

Four types of antigens have been successfully used. Instructions for their preparation follow.

a. Mouse spleen antigen. Inoculate 15 to 20 mice intraperitoneally with a very active virus and sacrifice 48 hours later. Triturate the spleens in a mortar with M/50 McIlvaine's buffered physiologic salt solution pH 7.6. It is most important that the spleens be rich in elementary bodies. Organs of mice which would have died on the 5th or 6th day after the inoculation of the virus yield suspensions of poor antigenic value.

Refrigerate the suspensions for 24 hours. The large particles are removed by centrifugation at 3,000 r.p.m. for 10 minutes, then the clarified supernatant fluid is centrifuged in an angle centrifuge for 1½ hours at 4,000 r.p.m. Discard the supernatant fluid, resuspend the sediment in buffer solution 1 ml. per spleen; then steam the suspension for 30 minutes in an ordinary Arnold sterilizer. The slightly opalescent solution may show sedimentation on standing and must be shaken before it is used.

b. Mouse lung antigen. Fifty mice are inoculated intranasally with 0 05 ml. of 10 per cent lung suspension of very active virus. If the mice have become prostrate after 45 to 48 hours, they are chloroformed. Remove the lungs aseptically and grind in a mortar with physiologic saline (pH 7.6), allowing approximately 2 ml. of saline per lung. Refrigerate the suspensions for 24 hours. After this period has elapsed, spin the supernatant fluid in a horizontal centrifuge for 10 minutes at 3,000 r p.m. to eliminate any remaining particles. Remove it carefully and centrifuge again for 1½ hours at 4,000 to 5,000 r p.m. in an angle centrifuge. The fluid is then discarded and the sediment resuspended in a physiologic salt solution to the original total volume. Shake it thoroughly; if particles still remain, centrifuge briefly in a horizontal centrifuge. Finally steam the suspension for 30 minutes in an Arnold sterilizer and store it in an icebox at 40° C. If the slightly opalescent solution shows sedimentation after storage, the particles must be resuspended before being used.

The same procedure is followed in preparing, from normal mouse organs, the antigens which are to be used as control antigens.

c. Culture antigen. Highly specific culture antigens may be made according to any of the modifications of the agar tissue method originally devised by Zinsser and Fitzpatrick for the cultivation of rickettsia. The semisolid agar tissue medium employed has the following composition:

Double Tyrode's solution with the following components:

Sodium chloride	160 gm
Potassium chloride	4 gm.
Calcium chloride	4 gm.
Magnesium chloride	2 gm.
Sodium acid phosphate	1 gm
Triple distilled water	1,000 ml
Sodium bicarbonate	20 gm
Dextrose	20 gm.

Phenol red indicator (stock solution):

Phenol red powder	0.1 gm.
N/20 sodium hydroxide	5.7 ml.

To prepare: Grind phenol red powder in NaOH, then add triple distilled water up to 250 ml.

Horse or beef serum	800 ml
Granulated agar	45 gm
Triple distilled water	1,200 ml

Extreme care must be exercised in the preparation of this medium or a heavy precipitate results. Mix the ingredients in the following manner:

(1), (2), (3), and (4) are prepared and sterilized separately.

(1) Dissolve 45 gm granulated agar in 1,200 ml. triple distilled water, autoclave 1½ hours at 15 pounds. Cool to 45° C.

(2) Filter cell-free horse or beef serum through Seitz filter . . . 800 ml.

(3) Dissolve in 300 ml. triple distilled water.

Sodium bicarbonate	24 gms
Sodium acid phosphate	1.2 gms.

Sterilize by filtration through Seitz filter.

(4) Dissolve in 900 ml. triple distilled water:

Sodium chloride	192 gm
Potassium chloride	4.8 gm.
Calcium chloride	4.8 gm

Magnesium chloride	2 4 gm
Dextrose	2.4 gm
Add phenol red indicator (stock solution)	64 ml.

Sterilize by filtration through Seitz pad.

Add serum to agar; then add (3) very slowly, mixing thoroughly. Finally, (4) is added and the medium dispensed.

When the temperature of each flask has reached 45° C., mix and pour into Kolle flasks or test tubes. Solidify and test for sterility.

Before they are used, 10-day chick embryos are beheaded and coarsely cut with scissors and then squeezed through the nozzle of a 20 ml. syringe. The infectious material (spleens of infected mice or infected yolk sac) is brought into contact with the minced embryonic tissue and after thorough mixing is kept in the icebox for ½ hour. Spread this material on the surface of the agar medium with a glass spatula, and tightly stopper the flask or tube, preferably with a rubber stopper. Elementary bodies usually appear in abundance in about 3 to 4 days Transplants are made by scraping off the cells with 3 ml. of saline and grinding the material in the pyrex hand grinder. After the coarse material has been permitted to settle, the supernatant is brought in contact with fresh chick embryo cells.

To prepare the antigen, remove the infected tissue from the agar surface and mix each flask with 50 ml. of buffered water (Sorenson's phosphate buffer pH 7.6 diluted 1.50). Place the suspension in a small metal Waring blendor (hermetically sealed) and triturate for 2 minutes Then centrifuge it in 25 ml amounts, in 50 ml. centrifuge tubes, at 3,000 r p m The supernatant fluid either may be subjected to repeated high-speed centrifugation at 15,000 r p m. or placed in an angle centrifuge at 4,000 r p m for 2 hours. Washed antigens prepared from the sediment are frequently not as antigenic as the crude preparations, which consist of the original suspension after centrifugation at 3,000 r p m The washed antigen generally has an infectivity titration end point of 10^{-7} to 10^{-8} when injected into mice by the intracerebral route To render suspensions noninfectious, heat in a steam sterilizer at 100° C. for 30 minutes. Preserve with 0 5 per cent phenol

Though laborious in preparation, the agar culture tissue antigens are rarely anticomplementary, are fairly stable, and have a high specific antigenicity. The loss of activity which results from storage must be compensated for by the use of less dilute antigens.[42]

d. Yolk sac antigen Inoculate 6- to 8-day chick embryos directly

through the air sac into the yolk sac. The suspension of psittacosis virus used is so standardized that the majority of the embryos die between the 56th to 72d hour after inoculation of 0 25 ml. of 1/30 dilution. Harvest the yolk sacs as soon as possible after death of the embryos and examine them for elementary bodies; if rich in virus, store in a dry-ice cabinet.

To make antigen, place the pooled yolk sacs in a heavy pyrex flask containing glass beads and shake vigorously in a small shaker for ½ hour Suspend each infected yolk sac in 8 to 10 ml. of McIlvaine's buffer solution pH 7 6 to which 0.3 per cent formaldehyde has been added. Store the suspension for 3 days at 5° C.

The noninfectious crude aqueous suspension is freed from the floating fat and coarse sedimented material by means of a separatory funnel. It is then centrifuged for 15 minutes at 2,000 r p m The aqueous midzone is placed in lusteroid tubes and spun in an angle centrifuge for 2 hours at 4,500 r p m, or for 30 minutes at 15,000 r p m, in a high-speed centrifuge Carefully resuspend the sediment in dilute buffered saline (4 volumes of 1.50 dilution of McIlvaine's buffer pH 7.6 + 1 volume saline solution) Shake the resuspended sediment thoroughly in a separatory funnel with an equal volume of ethyl ether and allow it to stand for 30 minutes at room temperature The heavy, opaque emulsion of egg material separates into the following components the moderately opalescent aqueous phase of a midzone of emulsion and a clear, faintly yellow layer of ether.

Remove the aqueous suspension and extract twice more with one-half volume of ethyl ether. Sedimentation of the elementary bodies is accomplished by centrifuging the extracts in lusteroid tubes in the angle machine at 4,500 r p.m. for 2 hours. Resuspend the sediment and centrifuge in an identical manner When a monodispersed elementary body suspension has been obtained the sediment is again resuspended in buffered physiologic saline solution in proportions equal to 3 ml per yolk sac These antigens, in a dilution of 1 4 to 1·8, fix complement in the presence of appropriate dilutions of serum derived from patients convalescing from psittacosis They are not anticomplementary, but lose activity upon storage, a disadvantage which may be compensated for by increasing the concentration of the stored preparations

An equally satisfactory antigen may be prepared by treating the crude suspension of yolk material with 0 5 per cent phenol. After hav-

ing been centrifuged to remove the coarse material and some of the fat, the suspension is boiled for 30 minutes. The material separates into a layer of fat and a precipitate after it has stood at 5° C. for several days; the intermediate turbid zone is then pipetted off and serves as an antigen.

Improvement in the preparation of yolk sac antigens appears to be possible in the light of the recent studies by Nigg, Hilleman, and Bowser [43] for lymphogranuloma venereum preparations and those of Topping and Shepard [44] for rickettsia.

e. Antigens from chorioallantoic fluid. When small amounts of antigens are required, the fluid of the incubated egg directly infected in the allantoic cavity with the virus yields a highly specific and active preparation.[43] Harvest the allantoic fluid 3 to 6 days after infection.

Before removing the fluid refrigerate the eggs for 12 to 18 hours; the yolk sac contracts, facilitating the aspiration of the clear fluid. A 10 ml. syringe is most satisfactory for aspirating. The fluid, which is rich in viral bodies, may be used as a crude antigen. As a precaution, however, it should be killed with 0.5 per cent phenol.

All 4 antigens have excellent keeping qualities when held in an ordinary refrigerator. They are titrated in 2-fold serial dilutions against a constant amount of specific human or pigeon psittacosis antiserum so diluted that 0.25 ml. contains 4 complement-fixing units.

f. Antigen titration.

Set up two series of tubes containing the following:

(1) 0.25 ml. of antigen in 2-fold dilution, 1:2, 1:4, 1:8, 1:16, 1:32, etc.

(2) To one set add 0.25 ml. of positive sera (dilute 1:10) previously heated at 56° C. for 30 minutes
To the other set add 0.25 ml. of negative sera (dilute 1:10) or 0.25 ml. of saline.

(3) To both sets add 0.5 ml complement (containing 2 full units).

(4) Incubate in a water bath at 37° C. for 2 hours.

(5) Add 0.5 ml of 2 per cent sheep cells containing 2 units of hemolysin to all tubes.

(6) Water-bath 37° C. for 1 hour.

The set containing positive serum gives the titration of antigen.

The set containing negative serum or saline gives the anticomplementary results.

The antigenic unit will be equal to the least amount showing complete fixation and not anticomplementary in that dilution. A suitable antigen will yield a titration of 1:32 to 1:256 or higher. Use 4 to 8 units in the test. Antigens of this group have not proved to be hemolytic, but a test is always run on each antigen. It is as follows:

(1) To 0.25 ml. of the antigen dilution to be employed in the test add 0.25 ml. of saline. Water-bath at 37° C. for 2 hours.

(2) Add 0.5 ml. of sensitized cells.

(3) Water-bath for 30 minutes at 37° C. It should show no hemolysis.

Inactivate serum at 56° C. for ½ hour. Prepare 2-fold dilutions with 0.85 per cent saline ranging in amount from 1:2, 1:4 to 1:512. Use higher dilutions if the end point is not reached.

3. *Hemolytic system.*

a. Sheep cells. After washing at least 3 times, use a 2.0 per cent suspension of sheep cells in 0.85 per cent physiologic salt solution. To titrate hemolysin use 0.5 ml. amounts of the 2.0 per cent suspension of sheep cells. For the test proper use 0.5 ml. which contains 2 units of hemolysin.

b. Hemolysin. Antisheep cell hemolysins are prepared as usual by immunizing rabbits with washed sheep red blood cells. Only sera of marked hemolytic properties (titer 1:1,000 or higher) should be used. In titrating hemolysins proceed according to Kolmer and Boerner.[11]

c. Titration of hemolysin. Prepare the following dilutions from a 1:100 dilution of hemolysin (2.0 ml. of hemolysin—98 ml. or 0.85 per cent saline): 1:1,000, 1:1,200, 1:1,600, 1:2,000; 1:2,400, 1:3,000, 1:4,000, 1:5,000, 1:6,000, 1:8,000, and 1:10,000.

Transfer 0.5 ml. of these dilutions in duplicate to 75 by 12 mm. tubes and add 0.3 ml. complement diluted 1:30, 1.7 ml. saline, and 0.5 ml. sheep red blood cells. Shake each tube thoroughly and water-bath for one hour at 37° C. The first tube which shows complete hemolysis is considered the end point of the unit. Two units of hemolysin are required in the test. Since 0.5 ml. has been used, the dilution to be employed in the test is that which is twice the concentration of the unit, e.g., if the unit is 0.5 ml. of 1:2,000 dilution, 2 units are contained in 0.5 ml. of the 1:1,000 dilution.

d. Complement. Pool the blood serum of at least 3 to 20 guinea pigs and distribute it in convenient amounts in pyrex tubes; seal and

keep frozen in the CO_2 cabinet. The titer will not drop for 6 months. Do not refreeze complement.

For titrating complement, prepare a 1:30 dilution by mixing 1 ml. of complement with 29 ml of 0.85 per cent salt solution. Pipette in duplicate the following amounts of the diluted complement: 0.10, 0 12, 0 14, 0 16, 0 18, 0 20 ml, etc. Then proceed in the following manner:

(1) Add sufficient sterile 0 85 per cent salt solution to obtain a total volume of 0.75 ml. in each tube For example: 0·15 ml of complement 1:30 + 0 6 ml of saline; or 0.2 ml of complement 1:30 + 0.55 ml. of saline, etc.
(2) Add 0 25 ml. of the dilution of the specific test antigen to be used in the test to one series; to the other series add the control antigen
(3) Incubate in a 37° C. water bath for 1 hour; then add 0 5 ml. of sensitized sheep cells which have been prepared 15 minutes previously.
(4) Incubate again for 1 hour and record.

The exact unit of complement is the amount which is contained in the 1st tube (gross amount of complement) showing complete hemolysis. The 2d tube showing complete hemolysis is the full unit Two full units (as defined by Kolmer) are used in the test.

4. *Technic of the complement fixation test with human and bird sera.* These tests are set up as serum titrations with a constant amount of antigen. Mix the reagents in the following order:

a. 0 25 ml serum dilution
b 0 25 ml antigen (diluted to contain at least 4 fixing units).
c 0 50 ml. complement (2 full units).

Incubate for 2 hours in a 37° C water bath. Then add 0 5 ml of 2 per cent sheep cells, containing 2 units of hemolysin Hold in water bath for 1 hour at 37° C, or for 30 minutes after control tubes clear Read the test, then place in the cold room overnight and record the final reading the following morning

Estimate the amount of fixation as ++++ (complete), +++ (75 per cent), ++ (50 per cent), + (25 per cent), ± (trace to 25 per cent), and 0 (none). Record as the titer the highest dilution showing ++++ or +++ fixation.

5. *Controls.*

a. Serum controls Add to duplicate tubes of the 4 lowest dilutions

of serum the same reagents used in the test, but substitute salt solution for the antigen.

b. Antigen controls Prepare antigen controls containing 0 25 ml in the same manner, but double the number of units used in the test (0 5 ml. of complement and 0 5 ml of sensitized cells). Antigens prepared from normal spleens or yolk sacs should be used. If suitable lymphogranuloma venereum antigens (Lygranum) are available, it is advisable to include them in the test series.

c. Hemolytic system Set up 4 tubes containing respectively, 0 05 ml, 0 1 ml., 0 15 ml, and 0.2 ml of the dilution of complement used in the test. These amounts represent ½, 1, 1½, and 2 units of complement. Bring up to a volume of 0 5 ml in each tube with sterile saline, and add 0.5 ml sensitized sheep cells The tube containing 0.05 ml. of complement (1 unit) should show + or ++ hemolysis and the 3 remaining tubes should be completely hemolyzed

d Standard serum Titrate a standard human or bird serum with each test, using the same dilutions as for the serum under test Serum from a patient recovered from proved psittacosis or that from pigeons known to be infected with the disease may be used as a standard serum. Such a serum, if moderately high in titer, is preferable to either a low titered sera or to one that is very high in titer; a serum in the titer 1 32 to 1 256 is considered satisfactory

A serum known to be negative should also be run

Results should be reported in the actual titers obtained It is imperative to procure more than one specimen from a patient, since the subsequent rise in titer during infection and convalescence is of diagnostic value *Low titers are not always significant.*

B. AGGLUTINATION TEST

For the serologic diagnosis of psittacosis infections in chickens and ducks, the test tube agglutination of elementary bodies may be attempted The procedure is not suitable for mass or routine examinations, since the preparation of suitable antigens is tedious and not very profitable [33]

1 *Preparation of antigens* Infected yolks are the source of the elementary bodies Purification is accomplished by differential centrifugation The infected yolk sacs are broken up to a final concentration of 20 per cent by being placed in a mechanical shaker for ½ hour with 0 004 M citrate-phosphate (McIlvaine) buffer *Five ml amounts of the emulsion are dispensed into 15 ml conical*

centrifuge tubes and spun at 2,000 r p m. for 10 minutes Discard the sediments containing the larger tissue fragments. Transfer the supernatants to 6 ml tubes and spin for 1 hour at 4,000 to 6,000 r p m. in an angle centrifuge Resuspend the sediments in buffer solution and centrifuge again at low speed At least 7 to 15 repeated washings are necessary to free the elementary body suspensions of tissue fragments.

Such suspensions are highly infectious The process of preparing them is dangerous when psittacosis agents of parakeet origin are used, and this work should be entrusted only to immune workers in special isolation rooms. The material is safe to handle when treated with 0 1 per cent formaldehyde

To prepare it for use, dilute the antigen in pH 7 0 phosphate buffered saline to an opacity twice that of a number 2 McFarland nephelometer.

2 Technic of test. In order to economize the available antigen, set up the test in small tubes. Serial 2-fold dilutions of the serum in 0.25 ml or less Add an equal volume of antigen to each tube, so that the final dilutions of serum range from 1·4 to 1 256 Normal and standard serum controls are included in each test, as is an antigen saline control Incubate the tubes in the water bath at 50° C. for 12 hours

Record the degree of agglutination as follows:

++++ = Complete agglutination, clear supernatant fluid (100 per cent)
++ = Agglutination definite, supernatant opalescent
0 = No agglutination

As a rule, the serums of infected birds will react in dilution 1 16 and higher.

C. COLD AGGLUTININS

Recent studies indicate that no significant titers of cold agglutinins are demonstrable in the blood of patients infected with the psittacosis virus[46] It is therefore advisable to conduct serum tests for cold agglutinins by the following method

Serial 2-fold dilutions of serum in saline in 0 5 ml. amounts, using 100 by 11 mm test tubes After adding equal volumes of 2 per cent red cells, shake the emulsion carefully and store overnight in the refrigerator The erythrocytes are obtained from group "O" donors and, as with oxalated blood, are carefully washed *in saline, cells not older than 2 to 4 days are used The racks of tubes* are usually placed in an ice water bath before the readings are made

The tests should not be read until the tubes have been rapidly inverted 3 times to loosen all of the cells from the bottom of the tube and to allow thorough mixing The agglutination is graded from 4+ to 1+, the former representing a single hard clump of cells not broken up on mixing, the latter indicating a finely floccular agglutination which is easily visible without magnification.

The titers are recorded as the reciprocal of the highest dilution of serum giving 1+ agglutination After the readings are made in the cold, the racks of tubes are placed in a water bath or incubator at 37° C for 2 hours All of the positive agglutinations recorded are then completely reversed by this procedure

D. INTERPRETATION OF LABORATORY RESULTS

1. *Isolation of psittacosis virus.* From the patient's standpoint, demonstration of the psittacosis virus in the blood or sputum is frequently of retrospective value, since the results are usually not available during the course of an acute illness. To the health officer, however, the etiologic recognition of certain forms of pneumonias during life or after death is very important. The proper identification of the viral agent after a bacterial cause is excluded may be of real assistance to the epidemiologist in locating the source of infection

Isolation of the psittacosis virus from the sputum of a person who lacks the clinical symptoms and signs of psittacosis suggests the existence of a latent infection or carrier stage. In this case, repeated examinations of sputum specimens are indicated.

The demonstration of the psittacosis virus in the spleen, liver, kidneys, and cloacal content of birds epidemiologically connected with the human infection possesses definite diagnostic value A flock of pigeons in which 10 to 20 per cent of the birds yield virus must be considered heavily infected and may serve under suitable conditions as a source of infection for human beings.

2. *Demonstration of complement-fixing antibodies.* The difficulty with the complement fixation test, aside from the preparation of an active antigen, lies in the interpretation of the results. Because of the ubiquity of viral agents which are capable of eliciting antibodies, and the long interval of time that these antibodies may persist, certain minimum criteria are essential in establishing proof that a positive serologic reaction is the result of recent infection It is now imperative to adopt the same criteria for the diagnosis of psittacosis that have been found necessary for influenza, lymphocytic choriomeningitis, encephalitides, etc [14]

The demonstration of complement-fixing antibodies in a titer of 1:8 (4+) in a single sample of a patient's serum is considered inadequate for a definite diagnosis. When accompanied by a good epidemiologic history, such a titer suggests that the patient may now be in the acute phase of a psittacosis infection or may in the past have been exposed to the virus Furthermore, as previously noted, the serums of patients suffering from lymphogranuloma venereum give cross complement fixation reactions with the psittacosis antigen Therefore, the complement fixation reaction must be supplemented by a Frei test In the absence of a positive Frei test, a positive complement fixation

with psittacosis or in many instances with Lygranum antigen may indicate the presence of psittacosis or an allied viral infection.

Complement fixation in a serum dilution of 1:16 (4+) or greater is considered highly suggestive of psittacosis or psittacosis-like infections. A 2-fold or greater rise in antibody titer during the course of an illness or in convalescence is considered significant only if the early and late samples of serum are tested in the same experimental series. Serums taken during the first 2 weeks of illness which give a complement fixation titer of 1:16—1:64+ + + + are suggestive of infection. To be conclusive, however, this must be followed by a second serum sample which shows a rise in titer. Serums taken after the 14th day should yield a titer of at least 1:32+ + + + or higher. The second test may show an increase in titer or remain the same. A serum titer of 1:8 or 1:16+ + + + during acute illness, which drops during convalescence is regarded as a nonspecific reaction.

The serums of patients with acute infections other than psittacosis may occasionally yield significantly high complement fixation titers, which fade during convalescence. This is in contrast to the psittacosis infections, in which the titer rises during convalescence and usually persists for many months and even years. These fleeting reactions, probably anamnestic, may result from the reappearance of antibodies indicative of a previous, unrecognized latent infection. Persons constantly exposed to psittacosis agents, such as aviary owners, pet shop employees, and pigeon breeders as a rule show complement-fixing antibodies in their serums in titers varying from 1:8 to 1:32+ + + + On the other hand, all the evidence thus far obtained indicates that in persons not previously exposed to the psittacosis virus, complement-fixing properties may appear in the serum as early as within a week after the onset of symptoms If the titer rises within the next 4 or 5 days, a tentative diagnosis of psittacosis may be rendered, and specific treatment instituted. However, additional serum specimens should be examined and the rise in titer noted No effort should be spared in attempting to isolate the virus from the sputum or the blood during the acute phase of the disease.

Additional precautions are necessary in interpreting the tests Patients' serums with positive Wassermann reactions may react strongly with the psittacosis antigen when they are simultaneously infected in a latent state with the virus of lymphogranuloma.[47]

The persistence of a high titer of complement-fixing antibodies in

the serum of a recovered patient at the end of 12 months suggests latent infection, with survival of the virus in the tissues. The demonstration of complement-fixing antibodies in titers of 1:4 (4+) and greater in the serums of psittacine birds and pigeons indicates either latency or recovery from infection. Birds actively shedding the virus as a rule have high titers, although pigeons with closed latent infections may have no complement-fixing antibodies in their sera At times, the viral agent cannot be isolated in birds with high titers.

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EPIDEMIOLOGY OF PSITTACOSIS AND ORNITHOSIS

HOUSE EPIDEMICS, OCCUPATIONAL AND ACCIDENTAL INFECTION

PSITTACIDAE

COLUMBIDAE

ANATIDAE

Microbacterium multiforme
(Filterable)

Incubation time - 7-31 days

High temperature with slight remissions

Toxemia entirely out of proportion to physical findings

Non productive pneumonia or pneumonitis

Sputum — Droplet or contact

Blood virulent - 1st-3rd day

Sputum virulent 5th - 23rd day

Regularly in fatal cases

Autopsy; virus in lung, spleen and liver

Complement fixation of serum — positive 8th-15th day

INFECTED DROPPINGS
DUST AEROGENIC ROUTE

Size of birds in relation to incidence and importance

Experimentally infected: Mice, Guinea Pigs, Hamsters, Pocket Gophers, Rabbits, Monkeys.

FIGURE 1

STEPS TO DETERMINE THE PRESENCE OF PSITTACOSIS VIRAL AGENT HUMAN SPECIMENS

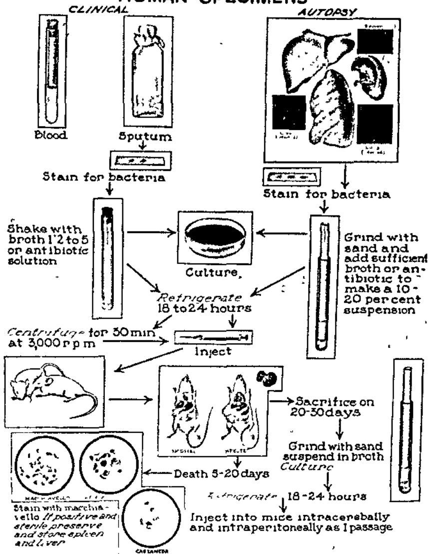


FIGURE 2

STEPS TO DETERMINE THE PRESENCE OF PSITTACOSIS VIRAL AGENTS INFECTED BIRDS (OR MAMMALS)

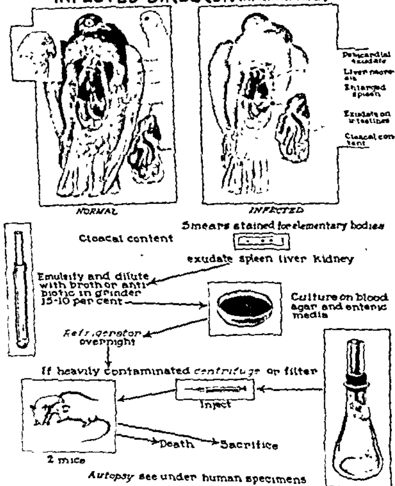


FIGURE 3

LYMPHOGRANULOMA VENEREUM

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I. INTRODUCTION

LYMPHOGRANULOMA VENEREUM, lymphogranuloma inguinale, or climatic bubo is a venereal disease. In its characteristic form it begins with a primary lesion—a small and transient vesicle on

the genitalia—which, especially in the female, is frequently unnoticed. This is followed in from 6 to 50 days by *painful swelling of the regional* inguinal lymph nodes, which will usually suppurate and drain through the overlying skin if left untreated. The whole episode usually runs its course without fever, but an acute febrile attack may be associated with the primary lesion, or a low grade and prolonged fever may complicate the adenitis.

A. CLINICAL FEATURES

More rarely the disease is protean in nature. There may be acute septicemic episodes at the onset, particularly in laboratory infections where dosage may be an important factor, and these may result in meningitis or pneumonitis. The primary attack may occur in the mouth, due to unnatural sexual practices or laboratory infections, and is sometimes highly destructive in this site; or in the eye also as a result of unnatural practices, where it produces a highly destructive disease distinguishable with difficulty from acute trachoma. Particularly in the female the spread of the disease to the pelvic lymph nodes, or a primary implantation in the rectum, leads to chronic elephantiasis of the genitalia, esthiomene, anal fistula, and rectal stricture.

The responsible agent *Miyagawanella lymphogranulomatis*[1] can be isolated from the primary lesion in the eye and probably from other *primary lesions if these are seen and recognized.* However, it is more usually obtained from the secondarily infected tissue—lymph nodes, anorectal tissue, or spinal fluid. It should be looked for in any case of inguinal adenitis without an adequate septic lesion on leg or foot even if a primary lesion on the genitalia, or the history of such, is missing Its presence should also be suspected in any case of chronic infection around the anus or rectum. The exclusion of other venereal diseases producing similar lesions is important. It may be pointed out that the disease though more frequent in Negro males is not limited to any race or sex.

B. THE AGENT

The usual form of the agent is a small coccoid body approximately 300 mμ in diameter, staining with aniline dyes, gram negative, and visible with the light microscope. Inside cells these elementary bodies may not be distinguishable as such since, particularly in the early stages of infection, they are enclosed within inclusion bodies. These

consist of a dense matrix derived either from the agent or from the cell—possibly from both. Particularly during the early stages of the infection, initial bodies approximately 1 μ in diameter are often found.

C. Pathologic Lesions

During the early stages of infection the characteristic lesion is that in the inguinale nodes. They become enlarged and matted together with a dense periadenitis. At a later date abscess formation is usual, and complicating hemorrhage may occur. On section there are found various stages of nonspecific inflammation even to complete destruction, but the typical lesions are small foci of epithelioid cells and some giant cells, not unlike tubercles or early gummata. These epithelioid foci are the site of multiplication of the agent and in them originate the abscesses which complicate the later picture of the lesion. Within the epithelioid monocytes may be found the characteristic inclusions within which, as has been indicated above, the elementary bodies are embedded.

II. ISOLATION AND IDENTIFICATION OF THE AGENT

A. Precautions for Workers

The agent is highly infectious for man. Laboratory infections have occurred through lesions on the hands and through the oral cavity.[3] Vaccination with such vaccines as have been available has not proved an efficacious prophylactic. In handling material suspected of containing this agent, particularly in high concentration, it is recommended that the workers wear goggles or a translucent face mask. Rubber gloves may be preferred but are likely to confer a false sense of security and to render the worker clumsier. It is well to bear in mind that the agent is considerably more resistant and stable than is indicated in the literature.

B. Collection and Preparation of Material

As stated above, the most common sources of material for isolation of the agent are bubo pus, biopsy material from inflamed node or anorectal tissue, and cerebrospinal fluid. When collecting any such tissues one should exert great care to avoid bacterial contamination. When such precautions are adopted spinal fluid should always be free of bacteria; tissue or pus from nodes free in 80 to 85 per cent of

instances[3, 4] *and anorectal tissue usually contaminated.* However, to ensure the highest proportion of positive isolations, aseptic technic, rather than indiscriminate use of sulfonamides or antiseptics, should be used. The specimens should be shipped in closely sealed glass containers within double mailing cases. Freezing the material is desirable but should be avoided unless maintenance of the frozen state can be assured until the specimen reaches the laboratory.

In the case of samples potentially contaminated with bacteria, further treatment is required before they can be used for inoculation. The agent of lymphogranuloma venereum is susceptible to commercial penicillin, but the concentrations required before any effect is observed (*in vitro* 2,250 u/ml.,[5] and *in vivo* 50 u/ml.[6]) are much higher than those required for antibacterial action. Also streptomycin in our experience has had no effect on the agent even with concentrations as high as 3,500 u/ml. *in vitro* or 20,000 u/ml. *in vivo*.[6] Material in which bacterial contamination is suspected may, therefore, be treated with final concentrations as high as 1,000 units of penicillin and 3,000 units of streptomycin per ml. *in vitro* before animal inoculation.

C. INOCULATION AND OBSERVATION

Two technics have proved most effective for animal inoculation—the intracerebral inoculation of mice and the injection into the yolk sac of chicken embryos. Although the latter method is more sensitive for the detection of small numbers of infective units of established strains, the greater susceptibility of the chicken embryo, compared with that of mice, to bacterial contaminants is a great disadvantage. Moreover, recent work has suggested that the intracerebral inoculation of mice is more sensitive in the isolation of new strains than is the yolk sac technic.[4] For both these reasons mice would seem to be the animals of choice in the inoculation procedure. Guinea pigs have also been used [7] but seem to offer no advantages over mice.

Most mice are susceptible to the agent inoculated intracerebrally, but the Swiss albino is markedly so and is suggested for use Care *must be taken to avoid stocks of mice spontaneously infected with Miyagawanella bronchopneumoniae*[8] (the agent of mouse bronchopneumonia—Gönnert[9]) or any other member of the family Chlamydozoaceae[8] related to the agent of lymphogranuloma venereum.

1 *Of animals.* Young 14 to 16 gm animals are preferred The sample, if of pus or tissue treated as suggested with penicillin and streptomycin, is prepared in

both 10 and 30 per cent suspensions in normal egg yolk by grinding with alundum Spinal fluid is used undiluted and diluted 1:10 Quarter ml. syringes with ⅝ inch 27-gauge needles are used. The mice are lightly anesthetized, the fur over the skull moistened with 70 per cent alcohol on a swab, and each of the animals then inoculated with 0.04 ml. with the needle inserted barely through the skull Five mice are inoculated with each dilution. They are then observed carefully at least twice a day for the next 10 days. Specifically infected mice will show on the 2d, 3d, or subsequent days ruffled fur, loss of weight, and a hunched-back gait. Especially in the first isolation passage nothing further may develop. Such mice should be killed, and the brains removed aseptically. Smears, or better impressions, of pieces of the cerebral cortex and meninges are made on a clean slide and stained with Macchiavello or Giemsa stain (see below). The rest of the brain is ground sterilely without abrasive, and a 10 per cent suspension is prepared in broth or normal yolk. This material is passed to 14 to 16 gm animals as above and into eggs by the yolk sac route If no sickness develops the brains of the mice are nevertheless passed on the 10th day to new mice and to eggs by the yolk sac route.

The number of "blind" passages to be made is open to question In our experience, however, if a strain of the agent of lymphogranuloma venereum is not established by the second passage, no strain has ultimately been derived no matter how many passages are made

2. *Of chick embryos.* For primary or subsequent inoculation into the yolk sac of chicken embryos, eggs of 6 days' prior incubation are chosen They are candled to ensure live healthy embryos, and the air sac is marked off A drill hole over the end of the air sac is made up to but not through the shell membrane. Material prepared as for intracerebral inoculation into mice is used, and 1 ml. of each dilution is inoculated into each of 5 eggs The eggs are candled twice a day. Deaths within the first 48 hours are almost certainly nonspecific or are due to bacterial contamination, but all are harvested, smears are prepared from the yolk sacs and stained (see below), and the yolk sacs are kept until a microscopic diagnosis is made All yolk sacs from eggs dying after 96 hours, if not bacterially infected, are passed in 10 per cent suspension in normal yolk to 3 normal embryos by the yolk sac route In our experience 2 passages have established all strains which will become established

D. Pathologic Specimens and Smears

1. *Preparation.* Smears or impressions of mouse cerebrum or embryo yolk sac are prepared on clean glass slides, they are then fixed with light heat by passing the slide rapidly several times through a Bunsen flame. Before making a smear of yolk sac, excess yolk should be removed by light impression on a gauze square Several stains are suitable, particularly Giemsa and Macchiavello. In general the latter has proved the more satisfactory and is used routinely.

2. *Stains.*

a *Giemsa stain for smears.* The smears are immersed in 95 per cent ethyl alcohol The slide is then flooded with Giemsa stain * diluted with equal parts 1·20,000 Na_2CO_3 solution. Warm slide to gentle steaming and allow to cool for 10 minutes. Wash vigorously with hot running tap water to remove precipitate *Blot dry.*

b *Macchiavello stain for smears.*

(1) 0 25 per cent solution of basic fuchsin in phosphate buffer M/5 pH 7.4 Grind fuchsin in mortar and add buffer gradually.

(2). 0 5 per cent citric acid solution

(3). 1 per cent aqueous methylene blue

The heated and cooled smears are flooded with solution *a* filtered through filter paper (Schleicher and Schull No 595) directly onto the slide After 4 minutes the stain is washed off with the citric acid Wash rapidly in water, stain for 10 seconds in methylene blue, wash in water and dry

Smears should be examined with a good oil immersion lens at a magnification not less than 900. The elementary bodies, *circa* 300 mμ in diameter, and the less numerous initial bodies, *circa* 1 μ in diameter, will be seen some within cells but for the most part lying free. With Macchiavello stain the elementary bodies are red (a few will be blue), and the initial bodies blue or red. Within the cells they often appear as dense clusters within vacuoles or embedded, and scarcely visible, within the matrix of inclusion bodies. When free and not very numerous, as is often the case with impressions of mouse brains, they will tend to occur in loosely scattered groups in one out of many fields. When more numerous, as from heavily infected yolk sacs, they are seen in pairs, chains, or densely packed morulae as well as singly.

Fixed specimens of brain or yolk sac may also be prepared for microscopic examination, but these are usually not as informative as are smears, and, furthermore, entail delay Noble's stain [10] is suggested as the most satisfactory for stained preparations, and, for this, formalin or, preferably, Helly's fluid fixation is required, with subsequent paraffin embedding.

c *Noble's stain for fixed tissues* Flood section with 1 per cent basic fuchsin solution and heat to steaming for 45 seconds Decolorize in 0 5 per cent citric acid 1½ minutes Wash with distilled water Counterstain with 2 per cent orange G for 5 minutes Rinse lightly and counterstain with 1 per cent methyl green for 5 minutes Differentiate rapidly in 95 per cent alcohol, pass rapidly through absolute alcohol into xylene, and mount in balsam

* Giemsa's spirochete stain from Hynson, Westcott, and Dunning, Baltimore, Maryland, has given satisfactory results.

3. *Examination.* In fixed preparations the agent is seen within cells except in those rare cases where a cell has recently burst. Elementary bodies are stained red with Noble's stain and may appear in dense clusters within vacuoles or may be embedded in the capsule-like matrix of the plaques, which stains green (For more detailed description of the microscopic appearance, see Rake and Jones[11]). Cell granules and cellular debris may cause trouble in diagnosis with the less experienced investigator, but the characteristic size, distribution, and staining of the bodies in heavily or moderately infected tissues is unlikely to be confused with anything else, particularly by those with previous experience. Pappenheimer, Molloy, and Rose have described the appearance in the yolk cells of granules produced by certain technics of fixation and staining, the nature of which is uncertain[12] These might cause confusion where encountered but normally do not do so.

Characteristic though the morphology and the staining may be, it is unfortunately shared by all the *Miyagawanellae* and even by the *Chlamydozoa* such as *C. trachomatis* or *C. oculogenitale* (agent of inclusion blennorrhea).[8] In order, therefore, that identification may be complete, the agent must be isolated. Even if the mouse is used for primary isolation, adaptation to the yolk sac of the chicken embryo should be carried out early. Yolk sac material is easier to handle and contains nearly a million times as much of the agent per gram as does mouse brain.

In those cases in which successful isolation is not achieved, positive diagnosis of the case from which the tissue came may be made through the inactivation of the material by heat or formalin and its utilization as antigen for skin tests in a known case or cases of lymphogranuloma venereum This is particularly easy with pus from buboes If the unknown sample contains inactivated agent or soluble antigen,[8] cases of lymphogranuloma will react to it in the same manner as to the antigens usually employed for skin test (see below).

E IDENTIFICATION

There are two principal ways in which specific identification can be obtained after an agent is isolated.

1 *Toxin-antitoxin.* The first is the toxin-antitoxin neutralization test[13, 14] *M. lymphogranulomatis*, in common with the other *Miyagawanellae* which have been studied, produces a specific toxic factor.[13, 14] The lethal effect of this on intravenous inoculation can be neutralized

specifically by homologous antitoxin produced in rabbits or in chickens. *Neutralization* can be carried out *in vivo* by passive transfer of protection, or *in vitro*. For practical purposes the latter is preferable.

Equal amounts of given dilutions of antitoxic serum in normal yolk and embryo fluids, and of toxin diluted in the same yolk and fluids to contain 4 M.L.D. per 0.5 ml. are thoroughly mixed and allowed to stand for 2 hours at room temperature. Twelve to 14 gm mice are inoculated intravenously with 0.5 ml. amounts of the mixtures. As controls and to determine the exact number of toxic units used, mice are inoculated with mixtures of equal parts of the same toxic suspensions and of the same dilutions of normal human or rabbit serum as those of the test sera. These control materials after standing at room temperature for 2 hours are further diluted 2-fold, 4-fold, and 8-fold in normal yolk and fluids and then are inoculated intravenously in 0.5 ml. amounts.

2. *Neutralization with chicken antisera.* The second specific method lies in the use of chicken antisera. Unlike rabbit sera, which fail to give satisfactory neutralization of the infectious activity of the *Miyagawanellae*, sera prepared by intraperitoneal inoculation of chicken give good neutralization and have a high specificity.[15]

Immune serum preparation * Following preimmunization bleeding, chickens are injected intraperitoneally 3 times a week with 3.0 ml. of 20 per cent saline suspensions of yolk sac or mouse lung heavily infected with *M. lymphogranulomatis* or other desired agent of the group. Five days following the last injection, the chickens are bled. Injections are repeated until the serums have satisfactory neutralizing antibody titers. Fifteen injections have been found sufficient although very few injections may give satisfactory sera.

Determination of neutralizing titer of immune sera. Undiluted immune serum is mixed with equal volumes of serial tenfold dilutions of heavily infected tissue beginning with a 1:5 dilution of tissue. Mixtures of infected tissue and normal serum should be included for control. Following incubation at 22° C. for 1 hour, 0.03 ml. of each of the mixtures is inoculated intranasally into six 7-10 gm. mice under light ether anesthesia.

Mice under test with *M. lymphogranulomatis* should be observed for 5 or 6 days, 10 days' observation has proved more desirable for other members of the group. All mice which die, as well as those which are still living at the end of the observation period, are examined for pulmonary lesions. The serum titer is expressed numerically as the difference between the activity of normal and immune serum in terms of protection based either on mean infectivity score[16] or LD_{50}. The infectivity score proved to be the more satisfactory method of expressing the titer because of the greater reproducibility of the numerical value.

Neutralization by the intracerebral technic is likewise demonstrable for those viruses which infect by that route. Groups of six 15 to 20 gm. mice are inoculated

* For this description the author is indebted to Dr. Maurice R. Hilleman.

intracerebrally with 0.03 ml. of the virus-serum mixtures described above and are observed for 21 days. Titer is expressed in terms of LD_{50} neutralized.

In a limited number of tests to date using the intracerebral technic, definite neutralization has been demonstrated. The intranasal test appeared to be more satisfactory, however, in that a greater numerical value for neutralization was obtained. Highly quantitative and readily reproducible results are obtainable by the intranasal method.

Hyperimmune chicken sera also give agglutination of homologous elementary bodies as do rabbit antisera,[24] but the specificity of such agglutination reactions remains to be determined.

3 *Other characteristics.* Quite apart from the above methods are others by which the agents of the *Miyagawanellae* may be distinguished. These depend on sulfonamide susceptibility and tissue tropisms.[3, 4] *M. lymphogranulomatis* is susceptible to sulfonamides as is also *M. bronchopneumoniae*[27] and some strains of *M. psittaci*.[24] (*Chlamydozoon trachomatis* and *C. oculogenitale* are not considered since they have not been grown outside the human host.) *M. psittaci* infects mice intraperitoneally and *M. lymphogranulomatis* does not; *M. bronchopneumoniae* fails to infect mice intracerebrally although *M. lymphogranulomatis* infects readily by this route.

F. VIABILITY OF AGENT AND PRESERVATION

The agent is remarkably stable. It can be kept at 0° to 5° C. for periods up to weeks in pus, in yolk, or in less suitable fluids such as broth or even physiologic saline, with little if any loss of activity. At −72° C. the stability is much greater, and in yolk no change in activity has been detected in 3 months at this temperature. Storage at −32° C. is less satisfactory probably because of slow changes to and from frozen to thawed state which occur at this temperature. Dried from the frozen state the agent has remained viable in our experience for 2 years with little change in activity.

III. IMMUNOLOGIC DIAGNOSIS OF THE DISEASE

A. SKIN TEST

The classical method of diagnosis of lymphogranuloma venereum depends on a sensitivity reaction, in the skin of infected persons, to an antigen containing inactivated agent. As originally described by Frei[19] this was carried out with inactivated human pus. Later the use of in-

specifically by homologous antitoxin produced in rabbits or in chickens. Neutralization can be carried out *in vivo* by passive transfer of protection, or *in vitro*. For practical purposes the latter is preferable.

Equal amounts of given dilutions of antitoxic serum in normal yolk and embryo fluids, and of toxin diluted in the same yolk and fluids to contain 4 M L D per 0 5 ml. are thoroughly mixed and allowed to stand for 2 hours at room temperature. Twelve to 14 gm mice are inoculated intravenously with 0 5 ml. amounts of the mixtures As controls and to determine the exact number of toxic units used, mice are inoculated with mixtures of equal parts of the same toxic suspensions and of the same dilutions of normal human or rabbit serum as those of the test sera These control materials after standing at room temperature for 2 hours are further diluted 2-fold, 4-fold, and 8-fold in normal yolk and fluids and then are inoculated intravenously in 0.5 ml. amounts

2. *Neutralization with chicken antisera.* The second specific method lies in the use of chicken antisera. Unlike rabbit sera, which fail to give satisfactory neutralization of the infectious activity of the *Miyagawanellae*, sera prepared by intraperitoneal inoculation of chicken give good neutralization and have a high specificity.[15]

Immune serum preparation * Following preimmunization bleeding, chickens are injected intraperitoneally 3 times a week with 3 0 ml of 20 per cent saline suspensions of yolk sac or mouse lung heavily infected with *M. lymphogranulomatis* or other desired agent of the group Five days following the last injection, the chickens are bled Injections are repeated until the serums have satisfactory neutralizing antibody titers Fifteen injections have been found sufficient although very few injections may give satisfactory sera.

Determination of neutralizing titer of immune sera. Undiluted immune serum is mixed with equal volumes of serial tenfold dilutions of heavily infected tissue beginning with a 1 5 dilution of tissue Mixtures of infected tissue and normal serum should be included for control Following incubation at 22° C for 1 hour, 0 03 ml of each of the mixtures is inoculated intranasally into six 7-10 gm mice under light ether anesthesia

Mice under test with *M lymphogranulomatis* should be observed for 5 or 6 days, 10 days' observation has proved more desirable for other members of the group All mice which die, as well as those which are still living at the end of the observation period, are examined for pulmonary lesions The serum titer is expressed numerically as the difference between the activity of normal and immune serum in terms of protection based either on mean infectivity score[16] or LD_{50}. The infectivity score proved to be the more satisfactory method of expressing the titer because of the greater reproducibility of the numerical value

Neutralization by the intracerebral technic is likewise demonstrable for those viruses which infect by that route Groups of six 15 to 20 gm. mice are inoculated

* For this description the author is indebted to Dr. Maurice R. Hilleman.

reactivity. Heating fully enhanced phenolized antigen in boiling water for 20 to 30 minutes increases its avidity for lymphogranuloma venereum serum without change in titer.

Inactivation of the agent determined by inoculating 0.6 ml of a 1.5 dilution of the 10 per cent phenolized suspensions in the yolk sacs of twelve 6-day embryos. The embryos are incubated for 12 days at 37° C. Deaths occurring within the first 2 days following inoculation are considered nonspecific. At least 10 of the 12 embryos must remain alive for 12 days after inoculation, and microscopic examination must rule out the presence of the agent.

Control antigens are prepared in identical manner from normal yolk sacs of embryos of about the same age as the infected embryos.

For use in the complement fixation test, stock antigen prepared as above is diluted to give 1 unit in 0.2 ml. or, if prepared personally, is diluted so that it is not anticomplementary, gives definite reactions with known weakly positive sera, and gives no reaction with known normal sera. Normal control antigen is diluted to the same extent.

Guinea pig serum (fresh or frozen) is used as complement. Just before use it is titrated and diluted so that 2 units are contained in 0.2 ml. This is usually obtained with 1:20 to 1:30 dilution.

To reduce anticomplementary behavior of human sera to be tested, these are inactivated at 56° or 60° C. for 20 minutes immediately before use. Tests are set up as serum titrations and 2-fold dilutions of the sera in physiologic saline are prepared, starting at 1:2.

The hemolytic indicator system consists of 0.2 ml. of a 3 per cent suspension of washed sheep red cells sensitized for 30 minutes at 37° C. with 2 minimal hemolytic doses of antisheep cell rabbit amboceptor.

The reagents in 0.2 ml. amounts are added to a series of tubes in the following order, diluted serum, 2 units of complement, specific antigen. A control series is set up in each instance in which normal yolk sac material replaces the specific antigen, and suitable controls are also employed to exclude anticomplementary or hemolytic action of antigen or serum (at the highest concentration tested) as well as to demonstrate the activity of the complement on the hemolytic indicator.

After incubation of 75 minutes in a 37° C. water bath, 0.2 ml of the hemolytic indicator is added. Readings for fixation (inhibition of hemolysis) are made after an additional incubation at 37° C. for 30 minutes. Only tests should be considered in which all controls are satisfactory.

The test has proved a more sensitive diagnostic method than is the intradermal test. That this is so can be shown in those cases which are followed from the earliest period following infection. Under these circumstances the complement fixation test becomes positive before

fected mouse brain was suggested.[20] Today the diagnostic material for the skin test is usually prepared from heavily infected yolk sacs.[21] The yolk sac antigen is standardized by prior testing on known positive and negative reactors and is diluted to give, in known positives, an intradermal reaction of suitable intensity. A control antigen is similarly prepared from normal yolk sacs.

Intradermal injections of 0 1 ml amounts of both specific and normal antigens are made into the flexor surface of the forearm. The reaction is read in 48 hours, at which time the central induration or papule is measured, and the surrounding erythema is ignored. Provided that no indurated or papular response to the normal antigen occurs, a papule of 6 mm or greater is significant

The yolk sac antigen has proved more sensitive than that prepared from mouse brain in the detection of the disease. The skin reaction is highly specific and does not occur in individuals with other diseases (with the possible and rare exception of some cases of pneumonitis due to other *Miyagawanellae*[22]) unless they are sensitive to yolk. Such individuals are identified by their reaction with normal yolk sac antigen

B. COMPLEMENT FIXATION TEST

More recently the complement fixation test has been widely used for diagnosis of lymphogranuloma venereum.[23] In this case inactivated material from heavily infected mouse lungs or embryo yolk sac, preferably the latter, is used. Other materials are not satisfactory because the concentration of specific antigen is too low.

In the earlier experiments infected yolk sac antigen and normal yolk sac control were treated with 0 1 per cent formalin in saline to inactivate the elementary bodies Other methods of inactivation have been suggested and that finally recommended makes use of phenol and is based on the original work of Nigg.[24, 25] It has the advantage of enhancing the activity of a given suspension of the agent and avoids, certainly to a large degree, those nonspecific reactions in early syphilis in which both specific and normal antigen give fixation,[26] and no diagnosis can be made. The method now employed by Nigg and Hilleman for preparing the antigen is as follows[27]

A suspension of yolk sac membranes heavily infected with *M lymphogranulomatis* is treated to contain 0 5 per cent phenol in a 10 per cent suspension of tissue Incubation of the suspension at 37° C for 3 weeks serves to inactivate the agent and to bring about a 16- to 32-fold enhancement of complement-fixing

been accomplished shows that such antibodies appear relatively soon after infection and are as specific as are the hyperimmune antibodies to the toxic factor produced in rabbits or chickens.[14]

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the intradermal and, in event of successful therapy—and also, one would presume, in spontaneous cure—the intradermal test will become negative before the complement fixation reaction. It is not clear whether these two reactions depend on different antibody-antigen reactions or whether the differences merely represent a quantitative difference in threshold. What evidence there is points to the former.[28, 29]

Three main sources of error occur, two of which can be excluded by the use of the control antigen and one of which cannot. Sera from persons sensitive to certain constituents of normal yolk or yolk sac will react nonspecifically with both specific and control antigens. There is also a group of sera from syphilitic individuals, in the early stages of the disease, which show a similar fixation with both antigens (see above). The use of phenolized enhanced antigen avoids most if not all of the latter nonspecific reactions. They disappear later in the course of the disease or following cure.

The third source of error occurs in individuals with latent or overt infection with other members of the family Chlamydozoaceae.[22, 30] Owing to the presence of an antigen or antigens common to all described members of the genera *Chlamydozoon* and *Miyagawanella*, fixation with sera from such cases will occur with specific and not with normal antigen. At the moment no methods have been evolved so to purify the antigenic complex as to remove the common antigen or antigens.

Apart from these three sources of error the test is highly useful. Many cases of latent infection are uncovered, particularly in venereally infected persons, even among those whose intradermal test is negative. Extensive epidemiologic studies are now possible so that sera collected for Wassermann, or other serologic tests for syphilis, may be utilized for such surveys It is not possible to say with certainty what level of serum dilution should be taken as the one beyond which a positive reaction indicates definite prior and continuing infection with some member of this group Opinions differ on this point. We believe it to be significant if fixation is obtained at dilutions of 1:4 or above.

C. NEUTRALIZATION OF TOXIN

One other test on sera from cases of lymphogranuloma venereum has been used and may prove to have practical value. This is the specific neutralization of the toxic factor by antibodies in the serum. Little investigation of this point has been carried out, but what has

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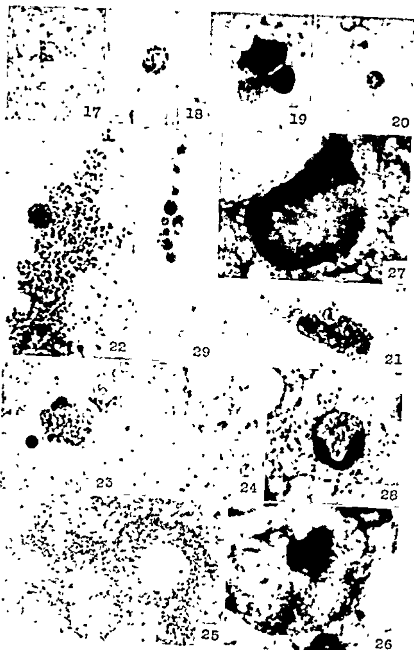

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Plate 1*

Smears of yolk sacs infected with the agent of lymphogranuloma venereum Giemsa stain Magnification × 1200

Figure 17 Group of initial bodies

Figure 18 Larger group of initial bodies

Figure 19. Group of elementary and initial bodies

Figure 20 Disintegrating plaque containing initial bodies

Figure 21 Disintegrating matrix with elementary and initial bodies

Figure 22 Disintegrating matrix with elementary and initial bodies

Figure 23 Disintegrating matrix with elementary and initial bodies Remnants of the matrix can be seen

Figure 24 Scattered elementary and initial bodies

Figure 25 Scattered elementary bodies

Figure 26 Intact yolk cell showing nucleus and many elementary bodies

Figure 27 Intact virus vesicle in a yolk cell

Figure 28 Monocyte with ingested elementary and initial bodies

Figure 29 Colonies of staphylococci from an infected yolk sac For comparison

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TRACHOMA

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III REFERENCES

C. THE VIRUS

The specific cause of trachoma is a virus of the psittacosis-lymphogranuloma venereum group of viruses. The taxonomy of this group of agents was long in dispute, but in 1945 Moshkovsky[2] suggested the family name of Chlamydozoaceae and the generic names of *Chlamydozoon* and *Miyagawanella*. The agent of trachoma he named *Chlamydozoon trachomatis* and the agent of inclusion blennorrhea, a closely related disease, he named *Chlamydozoon oculogenitale*. The virus of trachoma is believed to attack only conjunctival and corneal epithelium, and it is assumed that all subepithelial changes result from the presence of a diffusible toxin liberated by the virus in the epithelium. No one has been able to demonstrate the virus morphologically in subepithelial tissues, and no one has been able to infect the conjunctiva of the experimental animal except by way of the epithelium.

The virus appears typically in the form of elementary bodies morphologically identical with those of the other members of the psittacosis-lymphogranuloma venereum (Chlamydozoaceae) group. Just as also occurs in the course of the intracellular development of the other viruses of this group, large swollen forms appear, which are known as initial bodies and which have somewhat different tinctorial properties. The intracellular colonies of the virus, or inclusion bodies, are of diagnostic value in the human disease and need only be distinguished from the virus colonies of inclusion blennorrhea. The inclusion consists of the elementary and initial bodies embedded in a matrix made up principally of glycogen When stained with Giemsa's stain, or other similar dye, the young inclusions, consisting principally of initial bodies, tend to be more basophilic than the larger inclusions, consisting principally of elementary bodies. It is the elementary body which is the filtrable form, but filtrations are obtained with difficulty.[4]

Cultivation of the virus has not yet been obtained, although there has been no difficulty in growing its normal host cells, the conjunctival and corneal epithelium.[5]

D TRANSMISSION

The infectivity of trachoma is known to be very low in the chronic form of the disease but may be high in the acute form Transmission is from eye to eye and requires close contact The virus is quite sensitive to drying, and transfer tends to occur only under very unhygienic

I. INTRODUCTION

A. GENERAL STATEMENT

TRACHOMA is a virus disease of world-wide distribution which attacks the conjunctiva and cornea specifically, with serious sequelae leading to diminished vision or even blindness. The disease may start either acutely or insidiously, but if untreated always runs a chronic course over a period of many years. Cases are known to have lasted for more than fifty years, although it is true that when the disease affects young children there is a tendency to spontaneous cure in at least 30 per cent of the cases

B. CLINICAL FEATURES AND DIAGNOSIS

The most characteristic feature of the early acute phase of trachoma is a papillary hypertrophy of the conjunctiva, accompanied by an abundant conjunctival exudate in which neutrophilic leukocytes are predominant In its chronic phase follicular hypertrophy is the most characteristic feature. The cornea is involved simultaneously with the conjunctiva and eventually shows subepithelial infiltrates, pannus, and frequently corneal ulceration Cell necrosis with cicatrization is highly characteristic and useful in diagnosis. In severe cases conjunctival cicatrization may result in deformity of the lids, corneal cicatrization is the cause of the diminished vision or blindness A diagnostic clinical feature is the much more marked involvement of the upper parts of the conjunctiva and cornea than of the lower. In inclusion conjunctivitis, which is due to a virus very similar to trachoma virus, the maximum localization of the virus is in the conjunctiva of the lower lid

In most instances trachoma can be diagnosed on the basis of these clinical features alone, but laboratory procedures are distinctly valuable in difficult or complicated cases *Of greatest diagnostic importance clinically are (1) the follicular hypertrophy* involving the upper tarsal conjunctiva, (2) the development of pannus, and (3) the conjunctival and corneal cicatrization With the aid of the slit-lamp and corneal microscope, these characteristic changes can be recognized very early in the disease.

Secondary bacterial infection is very common in trachoma. In countries such as Egypt it may complicate the disease in 100 per cent of cases, the most important bacteria being *Neisseria gonorrhoeae* and the bacilli of the Hemophilus group In this country the most common secondary invader is *Staphylococcus aureus*, with *Diplococcus pneumoniae*, *Haemophilus influenzae*, and *H lacunatus* (diplobacillus of Morax-Axenfeld) next in order of frequency

Trachoma is favorably influenced by treatment with the sulfonamides, sulfanilamide and sulfadiazine being the drugs most commonly employed.

C. The Virus

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The virus appears typically in the form of elementary bodies morphologically identical with those of the other members of the psittacosis-lymphogranuloma venereum (Chlamydozoaceae) group. Just as also occurs in the course of the intracellular development of the other viruses of this group, large swollen forms appear, which are known as initial bodies and which have somewhat different tinctorial properties The intracellular colonies of the virus, or inclusion bodies, are of diagnostic value in the human disease and need only be distinguished from the virus colonies of inclusion blennorrhea. The inclusion consists of the elementary and initial bodies embedded in a matrix made up principally of glycogen. When stained with Giemsa's stain, or other similar dye, the young inclusions, consisting principally of initial bodies, tend to be more basophilic than the larger inclusions, consisting principally of elementary bodies. It is the elementary body which is the filtrable form, but filtrations are obtained with difficulty.[2]

Cultivation of the virus has not yet been obtained, although there has been no difficulty in growing its normal host cells, the conjunctival and corneal epithelium.[3]

D. Transmission

The infectivity of trachoma is known to be very low in the chronic form of the disease but may be high in the acute form. Transmission is from eye to eye and requires close contact The virus is quite sensitive to drying, and transfer tends to occur only under very unhygienic

conditions. The familial character of the disease is well demonstrated in such countries as North Africa and even among the Indians of our southwest. In these areas the father in the family may have the disease and the children remain free, but if the mother is infected the children are almost invariably also infected. Trachoma may spread simultaneously with bacterial conjunctivitis, particularly gonorrheal ophthalmia and Koch-Weeks conjunctivitis. In Egypt there has been some evidence to suggest transmission by flies, but this has by no means been proved. It is known that wrestlers are frequently infected, probably as a result of the close contact in wrestling and the common practice among wrestlers of massaging their opponents' eyeballs violently to induce a vagus reflex.

II. ISOLATION AND IDENTIFICATION OF THE VIRUS

A. Precautions for Workers

Unlike psittacosis virus, trachoma virus is not dangerous to work with, for its communicability is low, and ordinary hand precautions suffice to protect the worker. Accidental transmission of the disease to doctors and nurses has been known to occur but only during surgical procedures such as curettage and expression in which infected material has struck the eye. The wearing of glasses and the avoidance of contact of the hands with the eyes should provide sufficient protection for all practical purposes

B. Types of Procedure

Laboratory procedures in trachoma fall into two categories according to their purpose, as follows:

1. *For diagnostic purposes.*

a. Epithelial scrapings for the morphologic demonstration of the virus and the recognition of such complicating factors as allergy and tear deficiency.

b. Follicular expressions for the detection of specific cytologic changes.

c. Secretion smears and cultures for the determination of secondary bacterial infection.

2. *For experimental purposes.*

a. Animal inoculation (monkeys and apes).

As previously noted, the diagnosis of trachoma is primarily clinical,

only difficult cases requiring laboratory procedures. Examination of epithelial scrapings is first in order of diagnostic importance and examination of expressed follicular contents, second. Only in extremely acute cases can the virus be seen in secretion smears, but both smears and cultures are useful in the diagnosis of secondary bacterial infection. Knowledge of the presence or absence of secondary infection, and of its nature, if present, is a prerequisite to the application of adequate therapy.

Biopsy material is of little value in trachoma, although the virus can be seen in properly stained sections from the conjunctiva. For methods of staining sections the reader is referred to the chapters on psittacosis and lymphogranuloma venereum since the tinctorial properties of trachoma virus resemble very closely those of the viruses of these two diseases.

The inoculation of experimental animals has no diagnostic utility in trachoma. The technics are discussed in this chapter solely for the benefit of the experimental worker.

No serologic procedures of diagnostic importance have been developed as yet.

C. Preparation and Examination of Epithelial Scrapings

1. *Sources of material* The virus, which is to be found in both the conjunctiva and cornea, is most abundant in the early acute stages of the disease, and there is general agreement that much more of it is to be found in the conjunctiva of the upper tarsus and fornix than of the lower tarsus and fornix Epithelial scrapings from the upper tarsus and upper fornix, therefore, constitute the best source of virus material and the most important preparations from trachoma They are much better than biopsy material or follicular expressions since the virus appears to be limited strictly to the epithelium and is more abundant in the superficial layers than in the deep Scrapings are best taken with a platinum spatula from the previously anesthetized conjunctiva and from the area of greatest disease activity. This is usually the tarsal conjunctiva, and more particularly its upper border, but scrapings from the upper fornix are also valuable.

In the diagnosis of secondary bacterial infection, examination of scrapings from the lid margins is indicated when there is a complicating blepharitis.

2. *Staining methods* The scrapings should be placed on clean,

grease-free, dust-free slides. They are best fixed for 5 minutes with absolute methyl alcohol, acetone-free, and stained with Giemsa's stain. Wright's stain may be used as a substitute, but it is more difficult to obtain debris-free slides with this stain. Since the tinctorial properties of trachoma virus are almost identical with those of the other members of the psittacosis–lymphogranuloma venereum group of viruses, other stains such as the Castaneda, the Macchiavello, and the Victoria blue can be used, but they offer no real advantage and have been much less satisfactory than Giemsa's stain. For the technic of staining with the Castaneda and Macchiavello stains, the reader is referred to the chapters on psittacosis and lymphogranuloma venereum.

Giemsa's stain gives excellent, permanent preparations provided a reliable stain is employed and care taken in having a neutral distilled water as diluent. A stock preparation of Giemsa's solution can be obtained from various sources,* or the stock-staining solution can be prepared from powder † One-half gm. of the powder is dissolved in 33 ml. of the highest purity glycerol at 55° to 60° for 1½ to 2 hours. To this is added 33 ml. of absolute methyl alcohol, acetone-free. The solution is then mixed thoroughly and allowed to sediment overnight in a desiccator to prevent absorption of moisture. It is then poured off into small bottles and stoppered tightly.

Dilutions of stock Giemsa's stain are made with neutral distilled water or buffered water. If the water is allowed to become acid, the stain will shift to the red side, and if allowed to become alkaline, will shift to the blue. If there is any difficulty in obtaining satisfactory distilled water, a buffered neutral water can be used Distilled water is best kept in pyrex bottles, since ordinary glass bottles have a tendency to liberate alkali into the water. The preparation of buffered water is described in the chapter on psittacosis.

The fixed epithelial scrapings are stained as follows:

a Place the slides in a Coplin jar in a dilute Giemsa's solution (1 drop to 2 ml of neutral distilled water).

b. Place the jar in an incubator at 37° C. for at least one hour.

c. Remove stain debris by rinsing the slide rapidly in two changes of 95 per cent alcohol.

d. Dry and examine.

During the staining process care must be exercised to prevent dust

* Gradwohl Laboratories, St Louis

† National Aniline and Chemical Co, Inc, New York City.

particles from settling on the slide. If permanent preparations are desired, place a drop of cedar oil on the slide and cover with a thin cover slip. If more rapid staining is required, fix with May Grunwald solution for 3 minutes and stain with concentrated Giemsa's solution (2 drops to 1 ml. of neutral distilled water) for 15 minutes.

Wet fixation has been advocated by Lindner [4] for special studies of the inclusion bodies but it is not a diagnostic procedure. The iodine stain described by Rice,[5,6] which brings out the carbohydrate matrix of the inclusion body, has some usefulness, however, since it enables one to scan a slide rapidly under low power, the reddish brown glycogen color of the matrix being easily recognizable under low magnification. An inclusion body stained with one of the other dyes can more easily be missed unless oil immersion is used. It should be pointed out, however, that many of the small inclusion bodies do not have carbohydrate matrices and could, therefore, also be missed when stained with iodine. The number of inclusion bodies in any particular case seems to be in proportion to the clinical severity of the trachoma. As many as 20 per cent of the conjunctival epithelial cells in the pure, acute, exudative type may be affected whereas in chronic trachoma of very low intensity it may be necessary to search many slides before a typical inclusion body can be found. In such cases it is clearly helpful to scan the slides under low power magnification prior to the use of the oil-immersion lens.

3. *Morphology of the virus particles.* In epithelial scrapings from the affected conjunctiva or cornea, the virus appears in its more characteristic form, the elementary body, a minute coccus-like body about 0.2 to 0.25 μ in diameter, either singly, in clusters, or in clumps. The virus also appears in the form of initial bodies, which are larger, more bluish, bipolar-staining bodies, with coccobacillary morphology, and as intracellular masses of either elementary or initial bodies or both. The elementary bodies tend to stain purple with Giemsa's stain and the initial bodies, pure blue.

While the virus can be seen extracellularly in the acute stages of the disease, in the chronic stages it can usually be identified only in the form of the intracellular inclusion bodies. It is the inclusions, therefore, that have the greatest diagnostic value. As with other members of the psittacosis-lymphogranuloma venereum group of viruses, trachoma virus undergoes a definite sequence of morphologic change within affected cells. This sequence is believed to run as follows: An elemen-

tary virus particle is phagocytosed by an epithelial cell; the particle then swells to form a coccobacillary body many times its original size. This is the initial body, which then divides to form more bodies of comparable size. As the divisions continue, the cytoplasm of the cell is replaced with the inclusion mass, the elements of division become smaller and smaller, and finally the elementary body stage is reached again. From observations made during the incubation period of experimental trachoma in man, this sequence of morphologic change from elementary body through initial body and back to elementary body, with rupture of the cell and liberation of the virus particles, requires about 48 hours.

As previously pointed out, the inclusion mass is embedded in a carbohydrate matrix, which takes a characteristic glycogen stain with Lugol's solution. Finding of the typical cytoplasmic inclusion body limits the diagnosis to trachoma or inclusion blennorrhea. Differentiation between these two diseases, on laboratory evidence, must be based on cytologic differences rather than on any difference in the morphology of the inclusion bodies. The cytologic differences are best observed in follicular expressions, as will be discussed in Section D below.

4. *Morphology of the inclusion body.* Unfortunately, the trachoma inclusion body, which has such great diagnostic value, is not the only cytoplasmic element found in conjunctival scrapings Differentiation must be made from a number of pseudoinclusions. Among these is extruded nuclear material. This is usually easy to identify since the extruded material retains the tinctorial properties and characteristic texture of the original nucleus Other pseudoinclusions are phagocytosed nuclear material, pigment from heavily pigmented individuals such as the Indian or Negro, and stain debris. This last frequently simulates initial body inclusions, although the experienced observer can always make the differentiation. In view of these sources of possible confusion, however, the laboratory diagnosis of trachoma should, for all practical purposes, depend on the finding of the characteristic elementary body inclusion. This inclusion, with its typical elementary bodies (Fig. 1) and carbohydrate matrix (Fig 2) cannot be confused with any of the so-called pseudoinclusions.

As previously noted, the number of inclusions in pure trachoma varies directly with the amount of exudation Active, secreting trachoma, excluding those cases in which secondary infection is the cause of the exudation, always shows abundant inclusion bodies. Follicular

trachoma without much secretion, on the other hand, may have few, if any, inclusions.

5. *Recognition of complicating factors.* Epithelial scrapings are also useful in the determination of such complicating factors as allergy and tear deficiency. A high eosinophil count always indicates an allergy and most commonly a vernal catarrh The finding of keratinized epithelium in conjunctival scrapings is of importance as an indicator of tear deficiency, common in old cicatricial trachoma, and should always be checked by the Schirmer test, by which the output of tears in a measured period of time can be determined. Tear deficiency makes the eradication of secondary bacterial infection much more difficult.

D. PREPARATION AND EXAMINATION OF FOLLICULAR EXPRESSIONS

1. *Preparation.* As noted above, expressed trachoma follicle material shows specific cytologic changes which are of especial value in the differential diagnosis of trachoma and inclusion blennorrhea.[7] Preparations are best obtained by means of the Prince ring forceps or an ordinary chalazion curette. With one or the other of these instruments the material is gently expressed from a follicle on the anesthetized conjunctiva, placed on a clean slide, and stained with Giemsa's stain by the method described for staining epithelial scrapings

2. *Nature of the follicle* Diagnostically significant is the fact that the trachoma follicle, due to the characteristic necrotic changes which occur within it, is typically soft and gelatinous in contrast to the hard follicles of all types of follicular conjunctivitis and folliculosis. The trachoma follicle is, therefore, very easily expressed with the ring forceps, and the expressed material, when suitably stained with Giemsa's stain, shows a very characteristic cytologic picture In marked contrast to the easy expressibility of the soft trachoma follicle, the nontrachomatous follicle, such as that of inclusion conjunctivitis, is hard and difficult to express. In many instances the whole follicle must be literally torn from the conjunctiva This probably explains why follicular material from inclusion conjunctivitis presents a cytologic picture quite different from that of trachoma.

3. *Cytology of the expressed material* Examination of expressed follicular material from trachoma reveals the following picture (*a*) numerous macrophages, their cytoplasm loaded with broken-down nuclear material; (*b*) numerous pale-staining, seminecrotic, large mononuclear germinal center cells, probably lymphoblasts, which differ

sharply in appearance from the similar cells found in expressed material from nontrachomatous follicular conjunctivitis; (*c*) an extraordinary amount of cell debris scattered throughout the slide, and (*d*) a few scattered plasma cells and lymphocytes, typically far less numerous than the large mononuclear cells.

In expressed follicular material from inclusion conjunctivitis, on the other hand, the following changes are noted: (*a*) macrophages, when present, are very few in number and tend to be much smaller than the giant macrophages of trachoma; (*b*) there are no necrotic changes in the large mononuclear germinal center cells and little, if any, cellular debris; and (*c*) small round cells, mostly lymphocytes, predominate and constitute the most striking cytologic feature. This can be explained by the fact that in nontrachomatous follicular disease the whole follicle is torn out, whereas in trachoma only the necrotic central core or germinal center is expressed Under these circumstances the germinal center cells would be bound to be the most numerous.

E Preparation and Examination of Secretion Smears and Cultures

The cytology of the secretion in trachoma is significant only in the fact that the neutrophilic leukocytes are predominant, and that this finding, although also characteristic of inclusion conjunctivitis, differs sharply from the findings in follicular conjunctivitis caused by typical viruses such as epidemic keratoconjunctivitis virus and herpes simplex virus, both of which produce predominantly a mononuclear cell reaction.

The primary purpose of examining secretion smears and cultures in trachoma, however, is to determine the presence or absence of secondary bacterial infection or allergy There is indeed such an exceedingly high incidence of secondary infection in trachoma that smears and cultures should be made and examined routinely in order to ensure the application of adequate therapeutic measures This is particularly important in old cicatricial cases in which secondary infection may keep the trachoma active In such cases topical application of sulfathiazole, sulfadiazine, penicillin, or other chemotherapeutic agent may be very much more effective than their systemic administration

Secretion smears are best fixed by heat and stained by Gram's method When an acute bacterial infection is superimposed on a trachoma, the organisms most commonly seen will be the pneumococcus, bacilli of the Hemophilus group, and *Staph aureus* In rare instances *Strep viridans* or *Strep hemolyticus* may be found.

The occurrence of a conjunctival eosinophilia is indicative of a complicating conjunctival allergy, the most common type being vernal catarrh.

Routine cultures are best taken on blood agar plates by means of the wet swab technic (cotton applicators moistened with 1 per cent glucose broth) It is useful to divide the plate into four parts, two quadrants being used for each eye, one of the two for material from the conjunctiva, the other for material from the lid margins. Anaerobic cultures are usually not indicated. Special culture media are employed only in cases of suspected secondary infection with the gonococcus or the diphtheria bacillus, both of which are extremely uncommon complications in this country

F. Serologic Procedures

So far no serologic procedures of diagnostic significance in trachoma have been developed. Serologic findings have indeed been almost totally lacking,[8] due no doubt to the fact that trachoma virus is strictly epitheliotropic. It has so far been impossible to demonstrate viricidal antibodies in the blood serum, and second attacks of the disease occur without modification of symptoms. No hypersensitivity skin test, such as the Frei test for lymphogranuloma venereum, has ever been demonstrated. Rake, Shaffer, and Thygeson[9] found that serum from trachoma cases gave low complement fixation titers for lymphogranuloma venereum antigen, but their work needs confirmation. No trachoma antigen studies have been reported, but this is not surprising in view of the fact that trachoma virus has not yet been cultivated

G. Animal Inoculation for Experimental Purposes

1. *Source of material.* In animal experiments, epithelial scrapings taken from the area of greatest disease activity are best transferred directly without preliminary dilution Since the virus is never very abundant in scrapings, dilution tends to reduce its infectivity, but if it is necessary to transport the material or to make delayed inoculations, a concentrated suspension of epithelial scrapings in saline will usually remain active for periods up to 24 hours, particularly if kept on ice. At room temperature the virus is more rapidly inactivated[10]

2 *Selection of animals for inoculation* There has been considerable dispute as to which animals can be infected. It is now generally agreed, however, that only monkeys and apes contract experimental trachoma, and that the experimental disease is unlike the human disease in that severe complications such as cicatrization and pannus never occur In fact, experimental trachoma in monkeys is strictly a follicular conjunctivitis which runs a spontaneous course to healing, and bestows no

sharply in appearance from the similar cells found in expressed material from nontrachomatous follicular conjunctivitis; (*c*) an extraordinary amount of cell debris scattered throughout the slide, and (*d*) a few scattered plasma cells and lymphocytes, typically far less numerous than the large mononuclear cells.

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immunity. In no experimental animal is it possible, therefore, to make an unequivocal clinical diagnosis of trachoma. Furthermore, as has been repeatedly pointed out by such observers as Julianelle,[8] Wilson,[11] and Bland,[12] extreme care must be used in interpreting the results of monkey inoculations with trachomatous materials because of the animals' susceptibility to a spontaneous follicular disease which may simulate experimental trachoma. This disease, known as spontaneous folliculosis, has vitiated many experiments in trachoma and has made human inoculations necessary for final determinations. Closely resembling folliculosis in humans, the spontaneous disease consists of a follicular hypertrophy of the conjunctiva, particularly of the fornices, with minimal inflammatory signs.

The chimpanzee is by all odds the best experimental animal for trachoma, but the prohibitive cost of its purchase and care necessitates the use of monkeys or baboons for most research. The baboon (*Papio hamadryas*) has been more satisfactory than the *Macacus rhesus* since it is less susceptible to spontaneous folliculosis and develops an experimental disease easier to recognize clinically. Any animal selected for inoculation should first be examined carefully for evidence of the spontaneous disease.

3. *Routes of inoculation.* Direct surface inoculation of the conjunctiva is more satisfactory than subconjunctival injection. The material containing the virus can be rubbed lightly over the conjunctiva Light massage definitely facilitates infection, and even light scarification may help, although positive inoculations have been obtained as a result of simple instillation of the infectious material into the conjunctival sac.

4. *Experimental trachoma.* The incubation period of experimental trachoma in the rhesus monkey and baboon is usually from 1 to 3 weeks. The onset is always insidious, acute trachoma never occurring in the experimental animal, which should be observed for a full month after inoculation before the experiment is declared negative. As noted above, experimental trachoma is always self-limited, but its duration varies greatly from several months in the rhesus monkey to a year in the baboon. It is extremely difficult to demonstrate the virus morphologically in epithelial scrapings from experimental trachoma, but even when it is present in too small amounts to be seen, transmission is still possible.[13] Animals which have recovered from experimental trachoma are not immune to the disease, and a second infection appears to run very much the same clinical course as the first.



FIGURE 1 Epithelial cell with elementary body inclusion Giemsa stain



FIGURE 2 Same cell showing carbohydrate matrix Iodine stain



FIGURE 1 Epithelial cell with elementary body inclusion. Giemsa stain



FIGURE 2 Same cell showing carbohydrate matrix Iodine stain

INCLUSION BLENNORRHEA

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I INTRODUCTION

A. GENERAL STATEMENT

Inclusion blennorrhea,[1] or inclusion conjunctivitis, which is also known under a variety of names such as paratrachoma, genital trachoma, acute follicular conjunctivitis (inclusion body type), and swimming-pool conjunctivitis, is a virus disease whose principal manifestations are seen in the newborn baby It also occurs in the adolescent or adult, however, sometimes as a form of swimming-pool conjunctivitis

group. Like the trachoma inclusion, but in this case unlike the inclusions of the other viruses of the group, the inclusion blennorrhea inclusion has a glycogen-containing matrix. In general, inclusion blennorrhea virus is much more abundant in the lesions, particularly in the newborn baby, than is trachoma virus in the lesions of trachoma. This relative abundance of virus probably accounts for the ease with which filtrations[5, 6-8] have been accomplished in inclusion blennorrhea as opposed to the difficulty encountered with them in trachoma.

The pathologic lesions produced by inclusion blennorrhea virus are very similar to those produced by trachoma virus except that the necrotic changes in the cells of the subepithelial tissues which occur in trachoma, probably as the result of a soluble toxin liberated by the virus in the epithelium, are never produced by inclusion blennorrhea virus

All attempts to cultivate inclusion conjunctivitis virus have failed up to the present time. Unlike the other viruses of the group, neither trachoma virus nor inclusion conjunctivitis virus has been cultivable in the yolk sac of the developing chick embryo

So far no one has reported the preservation of inclusion conjunctivitis virus over a long period of time, although it should be possible to accomplish with such modern methods as desiccation *in vacuo* It is certain that inclusion conjunctivitis virus, like trachoma virus, is highly susceptible to drying Its viability when moist, on the other hand, is clearly indicated by its transmissibility[9] in the water of swimming pools At room temperature, however, it fails to remain active, even in the moist state, for periods longer than several hours Only at icebox temperatures will salt suspensions remain active for periods up to 24 hours.

D TRANSMISSION

Although *Chlamydozoon oculogenitale* is pre-eminently a cause of ocular infection, its main habitat is the genitourinary tract.[10] In the male it causes a benign urethritis, which heals spontaneously after a period of several months, and in the female it causes a type of cervicitis limited to a narrow strip of epithelium at the external os and to all practical purposes asymptomatic

Eye infections occur almost invariably as a result of transfer from the genitourinary tract to the eye. Eye-to-eye transmission[11] has been accomplished experimentally a number of times but has seldom

and again without any known source of infection. The disease is self-limited, running a course from acute onset through a chronic phase to healing in from 3 to 9 or 10 months. A few cases are on record in which the clinical manifestations persisted for more than a year

B. CLINICAL FEATURES AND DIAGNOSIS

In the newborn baby inclusion blennorrhea appears from the 5th to the 11th day after birth as an acute conjunctivitis characterized by great quantities of purulent secretion. The acute phase, which usually lasts from 10 days to 2 weeks, sometimes has alarming inflammatory manifestations, such as pseudomembrane formation. When these subside there is a chronic inflammatory phase which gradually resolves over a period of several months. There are no corneal complications and no sequelae except in those cases which have had marked pseudomembrane formation Some of these show fine conjunctival scars

In the adult the clinical manifestations of the disease differ so sharply from those in the newborn baby that at one time they were thought to constitute a different disease entity. Inclusion conjunctivitis in the adult[3, 4] is usually an acute follicular conjunctivitis without the abundant exudate so characteristic of the infection in the newborn Occasionally, however, a more severe form is seen in which papillary hypertrophy is predominant In this type the exudate is more abundant, although never in the amount seen in the inclusion blennorrhea of the newborn.

Unlike trachoma, which it resembles in many respects, inclusion blennorrhea cannot be diagnosed with certainty on clinical grounds alone Laboratory procedures are essential, the diagnosis being based on the finding of cytoplasmic inclusion bodies identical with those of trachoma. In the inclusion blennorrhea of the newborn, the finding of these inclusions is pathognomonic since in this country trachoma rarely occurs in the newborn baby and then never as an acute conjunctivitis It is the acute inclusion conjunctivitis of the adult that must be differentiated from trachoma The clinical findings then complete the picture since the virus of inclusion conjunctivitis does not produce corneal changes, and trachoma virus invariably does Laboratory differentiation based on the cytologic picture of expressed follicular material is also possible, as was described in the section on trachoma

C. THE VIRUS

The specific cause of inclusion blennorrhea is a virus of the psittacosis-lymphogranuloma group of viruses, now know as *Chlamydozoon oculogenitale*[4] and identical morphologically with trachoma virus Its intracellular colonies or inclusion bodies cannot be differentiated from the inclusion bodies of trachoma, and it undergoes the same intracellular cycle of morphologic variation (Fig. 1) as trachoma virus and, indeed, as the other viruses of the psittacosis-lymphogranuloma venereum

group. Like the trachoma inclusion, but in this case unlike the inclusions of the other viruses of the group, the inclusion blennorrhea inclusion has a glycogen-containing matrix. In general, inclusion blennorrhea virus is much more abundant in the lesions, particularly in the newborn baby, than is trachoma virus in the lesions of trachoma. This relative abundance of virus probably accounts for the ease with which filtrations[4, 6-8] have been accomplished in inclusion blennorrhea as opposed to the difficulty encountered with them in trachoma.

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Eye infections occur almost invariably as a result of transfer from the genitourinary tract to the eye. Eye-to-eye transmission[11] has been accomplished experimentally a number of times but has seldom

occurred spontaneously In the newborn the infection is transferred from the cervix of the mother to the baby's eye during birth; in the adult it results from a transfer of genitourinary material to the eye, either directly by way of the fingers or indirectly through water as in the case of swimming-pool infections. The epidemiology is strikingly similar to that of gonorrheal conjunctivitis. In fact the only difference is the swimming-pool transmission, which seems to be based on the fact that although both agents are highly susceptible to drying, inclusion conjunctivitis virus is considerably more viable when wet than is the gonococcus.

II. ISOLATION AND IDENTIFICATION OF VIRUS

A PRECAUTIONS FOR WORKERS

Workers with inclusion blennorrhea virus need only exercise ordinary caution to avoid accidental infection. When scrapings or expressions are being taken from patients or experimental animals, the wearing of glasses should prevent the spattering of infectious material into the eyes. This is particularly important when working with the newborn baby since dammed-up secretion very often spurts suddenly from the eyes when the lids are opened The worker should avoid rubbing his eyes with his fingers while working with the disease, but ordinary washing of the hands with soap and water is sufficient to remove the virus

B TYPES OF PROCEDURE

The following laboratory procedures are useful for diagnostic and experimental purposes in inclusion blennorrhea·

1. *Epithelial scrapings* for morphologic demonstration of the virus.
2. *Follicular expressions* for cytologic studies and differential diagnosis from trachoma
3. *Secretion smears* for diagnosis in acute cases and in inclusion urethritis in the male
4. *Animal inoculations* for experimental purposes and in the diagnosis of inclusion urethritis in the male

As with trachoma virus, the strictly epithelial localization of inclusion blennorrhea virus makes epithelial scrapings much more effective for its morphologic demonstration than are follicular expressions. The latter are useful, however, for cytologic studies and for dif-

ferentiating inclusion conjunctivitis from trachoma on the basis of cell reaction. For a description of the method of preparing follicular expressions and of the cytology of the expressed material from both inclusion blennorrhea and trachoma, the reader is referred to the section on trachoma.

Secondary bacterial infection is uncommon in inclusion conjunctivitis and of no significance in its management, although before the Crédé method of prophylaxis came into general use cases of mixed inclusion blennorrhea and gonorrheal ophthalmia were occasionally seen. Routine examination of secretion smears and cultures are, therefore, of only secondary importance. In acute cases smears may suffice for diagnostic purposes, but epithelial scrapings are always to be preferred. Smears have special utility in the diagnosis of inclusion urethritis, however, since urethral scrapings are usually impossible to obtain.

Animal inoculations are made principally for experimental purposes but have some practical utility in the diagnosis of the genitourinary disease, especially the nonspecific urethritis in the male in which microscopic diagnosis is difficult.

C. Preparation and Examination of Epithelial Scrapings

1. *Sources of material.* The best source of inclusion blennorrhea virus is pooled epithelial scrapings from cases of inclusion blennorrhea in the newborn. Although this is the best source of a high concentration of virus, less highly concentrated virus-containing material can also be obtained from inclusion conjunctivitis of the adult, more abundantly from the papillary type, and from the genitourinary disease in both male and female As it is usually not possible to obtain urethral scrapings in the male, the urethral exudate must be examined instead The virus is often in such low concentration in the exudate that it is quite difficult to see it in smears, although the same material will usually be infectious for baboons Cervical infections, on the other hand, yield epithelial scrapings which frequently show a high concentration of virus.

As was mentioned in the section on trachoma, the two viruses differ with respect to their maximum localization, trachoma virus tending to localize in the conjunctiva of the upper lid predominantly and inclusion blennorrhea virus in the conjunctiva of the lower lid predominantly. Inclusion blennorrhea scrapings should, therefore, always be taken from

the conjunctiva of the lower lid and fornix and during the period of greatest disease activity; that is, the early acute stage which usually lasts from 1 to 2 weeks. The amount of virus diminishes rapidly as the disease progresses.

As noted above, inclusion blennorrhea virus *in normal salt solution* can be kept refrigerated for periods up to 24 hours. Under ordinary circumstances, however, material should be used immediately after its collection.

2. *Staining methods.* Inclusion blennorrhea virus has the same tinctorial properties as the other viruses of the psittacosis-lymphogranuloma venereum group. While Giemsa's method, described in detail in the section on trachoma, has been used more widely in the study of this virus than any other method, Wright's stain has also been employed with success, and the reader is referred to the chapters on psittacosis and lymphogranuloma venereum for descriptions of this and a number of other elementary body stains which have been used in the study of various members of the group.

The iodine stain, which brings out the carbohydrate matrix of the inclusion body, has less utility in this disease than in trachoma, since the inclusions are usually sufficiently numerous to be picked up readily in Giemsa-stained preparations under high magnification. The glycogen staining of these two types of inclusion bodies, however, is interesting as the chief property differentiating them from the inclusions of other viruses of this group.

3. *Morphology of the virus* Inclusion conjunctivitis virus is indistinguishable morphologically from psittacosis virus, lymphogranuloma venereum virus, and the other viruses of the group. In Giemsa-stained smears of exudate from acute cases it appears in the form of elementary and initial bodies, a combination which gives a picture so characteristic as to be diagnostic. In Giemsa-stained epithelial scrapings the virus is seen intracellularly as inclusion bodies (Fig. 2), ranging in size from minute inclusions containing only a few virus particles to mature inclusions which may replace the entire cytoplasm of the cell. In very severe cases as many as 20 per cent of the epithelial cells may be infected. Multiple infection of epithelial cells also occurs, sometimes as many as 3 or 4 inclusions developing in a single cell. Like the trachoma virus inclusion, but unlike the inclusions of the other viruses of the group, the matrix of the inclusion body of inclusion blennorrhea consists at least in large part of glycogen which takes a reddish brown stain with Lugol's solution.

D. ANIMAL INOCULATIONS

1. *Experimental inclusion conjunctivitis.* Like trachoma virus, inclusion conjunctivitis virus has been transmitted only to monkeys and apes. Unlike experimental trachoma, however, experimental inclusion conjunctivitis (Fig 3) resembles closely the human disease, particularly the acute follicular conjunctivitis of the adult. Thus, inoculation of the conjunctiva of the baboon with infectious material results, after an incubation period of a week to 10 days, in a follicular conjunctivitis, rather subacute in character, which runs a course over a period of several months and heals spontaneously without residuals During the first few weeks of the experimental disease, the virus is readily demonstrable in epithelial scrapings. As the disease progresses virus demonstration in scrapings becomes increasingly difficult, but the existence of the infection can be established by transfer of the disease to other experimental animals. Like experimental trachoma, experimental inclusion conjunctivitis heals spontaneously without immunity. Baboons, for example, have been infected with the virus repeatedly, and the disease has run a characteristic course with each succeeding inoculation without significant modification

Animal inoculation appears to have no practical significance except in the diagnosis of the genitourinary disease, particularly of the nonspecific urethritis in the male. Owing to the small amount of virus in the exudate, as well as to the difficulty of obtaining epithelial scrapings from the urethra, baboon inoculation has been found to be a practical procedure.

2. *Sources of confusion.* As in the study of trachoma by animal inoculation, the main source of confusion is the presence of a spontaneous folliculosis which may simulate experimental trachoma or experimental inclusion conjunctivitis. Fortunately, the skilled observer finds the similiarities not too great Experimental inclusion conjunctivitis, with its subacute onset and characteristic course, combined with the presence of inclusion bodies in epithelial scrapings in the early stages of the disease, is rather readily differentiable from the spontaneous folliculosis Every effort should be made, however, to obtain animals which are free from the spontaneous disease

In inoculation experiments on the baboon the only virus diseases to be ruled out are trachoma and lymphogranuloma venereum Unfortunately, there is at present no known way to distinguish experimental trachoma from experimental inclusion conjunctivitis except by human inoculation, since both diseases in animals are essentially an acute follicular conjunctivitis without pannus or scarring

Lymphogranuloma venereum virus might cause confusion, since it is occasionally found in nonspecific urethritis Experimental lymphogranuloma venereum conjunctivitis of the baboon, however, bears little or no clinical resemblance to the acute follicular conjunctivitis of inclusion blennorrhea in baboons. Another way of distinguishing these 2 viruses would be by means of the differential staining of the inclusion bodies. The inclusion body of lymphogranuloma venereum does not have the carbohydrate, glycogen-staining matrix characteristic of the inclusion bodies of inclusion conjunctivitis and trachoma.

E. SEROLOGIC PROCEDURES

Inclusion conjunctivitis virus has no more ability to induce immunologic reactions than has trachoma virus. There is no hypersensitivity skin reaction and, except for the presence of group complement-fixing bodies as demonstrated by Rake, Shaffer, and Thygeson,[12] there have been no serologic findings. These complement-fixing bodies are of low order and have no role in the laboratory diagnosis of the disease.

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Developmental Cycle of Inclusion Body of Inclusion Blennorrhea.

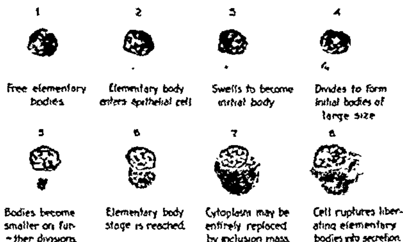
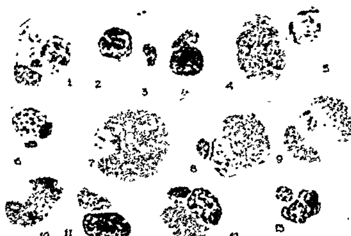


FIGURE 1 Developmental cycle of virus of inclusion conjunctivitis Schematic drawing



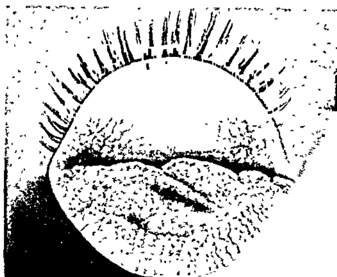


FIGURE 3 Experimental inclusion conjunctivitis of the baboon

VARIOLA AND VACCINIA

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I. INTRODUCTION

VARIOLA shows the clinical features of the acute infectious diseases and is distinguished by a characteristic eruption which appears as a rule after the peak of initial fever has been passed.

The onset is usually abrupt, with malaise, myalgia, headache, and backache. The fever tends to be high and well sustained for about 3 days. During this initial febrile period rashes of various sorts may appear, in some cases resembling the purpuric eruption of meningococcal infection. In about 3 days the temperature falls, in a mild case to normal, and about this time the characteristic eruption appears. This is macular at first, but soon becomes papular, then vesicular, and finally pustular. The lesions when discrete tend to be circular and typically

are loculated. This characteristic, however, should not be relied upon too heavily, nor should the fact that the eruption normally appears at one time so that the lesions are all in the same stage of evolution. The lesions originate in the malpighian layer of the skin and are therefore deep-seated. The eruption tends to favor exposed and traumatized surfaces, so that the face is usually involved, and it is heavier on extensor than flexor surfaces and on distal rather than on proximal parts of extremities. Visceral lesions usually are minor and not characteristic of the disease.

The eruptive lesions are rich in virus, which is fairly resistant to destruction. The infection may, therefore, be transmitted by droplets, virus entering respiratory secretions from lesions of the mucous membranes, by contact, by infected fomites, and by dust containing the dried virus. Man is usually highly susceptible to infection, but solid immunity is conferred by recent vaccination.

Vaccinia is a related infectious disease of cattle, its artificial transmission to man being designated as vaccination or smallpox vaccination The strains which are being most widely transmitted at present cause mild illness, the first symptoms of malaise and myalgia appearing about 4 to 5 days after inoculation These symptoms and the fever, which is usually moderate, reach their peak about the 10th day and then subside The eruption normally is confined to the site of inoculation and actually represents a localized, confluent mass of pustules, not distinguishable anatomically from those of variola The diagnosis of vaccinia is normally self-evident, confusion arising when the inoculation is accidental or when the eruption is generalized, as it is infrequently, or when the lymph is accidentally inoculated in an unusual location The confusion caused by a generalized eruption is obviously greater when the vaccinated individual has also been exposed to smallpox. Final differential diagnosis in this case can only be made by isolation and identification of the virus

The laboratory diagnosis of infection with either smallpox or vaccinia is best made by direct demonstration of the virus or its antigens in material derived from the patient. The characteristics of the virus and the richness of the cutaneous lesion in both virus and soluble antigens combine to facilitate it. On the other hand serodiagnosis requires waiting until the patient is convalescent and has developed specific antibodies. Further, the results are often vitiated by recent or remote vaccination.

II. ISOLATION AND IDENTIFICATION OF VIRUS

All persons actually engaged in work with the virus of variola and all others in the laboratory should be revaccinated even though they have been vaccinated as children, and perhaps since If this procedure is repeated each time the virus is reintroduced into the laboratory and if care is taken to avoid accidental inoculation of the corneas of workers, the virus can be handled with impunity. Normal cleanliness should suffice to keep the virus from leaving the laboratory accidentally.

There are three methods available for isolating or identifying the virus These are: (1) staining and direct identification of the elementary bodies, (2) inoculation of the rabbit cornea; and (3) inoculation of the chick embryo.

A. DIRECT IDENTIFICATION OF THE ELEMENTARY BODIES

For direct demonstration of the elementary bodies of the virus, the technic of van Rooyen and Illingworth[1] is recommended

The method which is now given requires clear microscopic slides, a scalpel, Loeffler's tannic acid flagellar mordant (20 per cent aqueous solution of tannic acid, 100 ml, saturated solution of ferrous sulfate, 50 ml; and saturated alcoholic solution of basic fuchsin, 20 ml; mixture to stand for 3 days and to be filtered before use), and dilute carbol fuchsin stain. (Two stock solutions stored separately, consisting of (a) 0.3 gm basic fuchsin in 10 ml. ethyl alcohol, and (b) 5 gm phenol dissolved in 95 ml distilled water One part of solution a and nine parts of b are mixed together and filtered before use)

Several early lesions are selected, preferably fresh vesicles containing clear fluid, but papules and pustules may be used The tops are carefully removed (trying to avoid drawing blood in the case of papules), and any fluid exudate is gently blotted with a dry, sterile, cotton pledget The base of the lesion is firmly scraped with a sharp scalpel, and the material smeared on the microscope slide It is well to place the scrapings of about three lesions on each slide, and to prepare at least two slides from each case

The slides are permitted to air-dry, and then are placed upright in distilled water for 2 to 15 minutes according to thickness, and then again allowed to dry in air They are then flooded with ether for 1 minute, and then with alcohol for 2 minutes and allowed to dry Slides are infectious prior to this treatment Freshly filtered mordant is added, the films are heated very gently, and the mordant allowed to act for 3 minutes Slides are then washed repeatedly and very thoroughly with running water for 2 minutes. The freshly filtered carbol fuchsin stain is poured on and gentle heat applied until steam rises, the films are then left for 7 to 10 minutes and then washed well with water They are dried, blotted, and examined For permanent preparations, they are mounted in neutral Canada balsam before examination

Stained according to this method, the viruses of variola and of vaccinia appear as clumps and masses of elementary bodies, well stained with fuchsin and about 0.25 μ in diameter. They may be confused with elementary bodies of varicella, but these usually occur singly, are present in small numbers, and are much smaller than those of variola. A diagnosis should only be made if the bodies are present in large numbers, and in clumps and masses as well as singly.

B. INOCULATION OF THE RABBIT CORNEA

For inoculation of the rabbit cornea,[3] the infectious material may be taken directly on the scalpel to be used for the inoculation, in a glass capillary tube from vesicle or pustule, or as dried crusts from older lesions. The method to be used for collection of fluid and of crusts is described in the next section.

If the laboratory is conveniently situated, it is simplest to take the material from the lesion on a scalpel, which may then be used for the animal inoculation. In order to obtain the virus, the skin bearing a well-developed pustule is cleansed lightly with ether, the lesion is then opened, and the fluid which is expressed is collected on the edge of the knife. If the lesion is a macule, its top is removed, the base is gently curetted, and the scrapings allowed to dry on the knife edge. The instrument is then placed in a sterile tube for transportation to the animal room. The rabbit to be used should be in good health, and not previously exposed to vaccinia or variola. The inoculation is made preferably under general anesthesia, although local anesthesia of the eye (2 per cent solution of procaine or metycaine instilled in the conjunctival sac) *may be used satisfactorily. In either case the head of the rabbit is fixed,* held either by an assistant or in a suitable holder, with the left eye up. A smooth retractor (handle of a scalpel) is gently pressed on the lower lid, as though to enucleate the eye. This pushes the eye forward in its socket and fixes it. The infectious material is then rubbed onto the surface of the cornea, 3 parallel incisions are made lightly in a perpendicular and 3 in a horizontal axis, and the infectious material is also rubbed into the light scarifications A sham inoculation should be made in the cornea of the right eye, using a sterile instrument. If a suspension of suspected material is being used, the eye is anesthetized and scarified as described. One or 2 drops of the fluid are dropped on and rubbed over the cornea. The eye is then allowed to fall into its normal position, and an additional 2 or 3 drops of material placed in the conjunctival sac. The eye is then closed, and the eyelids are rubbed gently over the cornea with the fingers.

A positive result is indicated by the appearance within 48 to 72 hours of a specific keratitis, characterized by the presence of a number of crateriform lesions along the line of inoculation, as well as a more diffuse corneal clouding in many cases.

If keratitis appears, the animal should be sacrificed in 72 hours after inoculation, the eye removed and fixed, the cornea sectioned, stained with hematoxylin and eosin or eosin-methylene blue, and the cells searched for the presence of Guarnieri bodies. These are acidophilic cellular inclusions, and their presence is required for conclusive diagnosis. In skilled hands, this method will yield a positive result in from 50 per cent to over 90 per cent of proved cases of smallpox. A negative result is, however, not conclusive.

C. INOCULATION OF THE CHICK EMBRYO

The chick embryo is the animal of choice for isolation of the virus.[8] It is susceptible to infection with both variola and vaccinia viruses, giving characteristic reactions to each. It is inexpensive, readily available, and easily manipulated.

For inoculation, the virus must be prepared in a watery (saline) suspension. This may be prepared by washing the scrapings from the base of a papule from the scalpel into a small quantity of physiologic saline solution. It is better prepared, however, from vesicular or pustular fluid, or from the dried crusts of older lesions. Vesicular fluid is most easily collected in capillary tubes, and these also form convenient containers for shipment of the virus. The lesion is cleansed by wiping lightly with an alcohol pledget, and then opened either with a scalpel or with the tip of the capillary itself If gentle traction is exerted on the skin the liquid contents of the vesicle will usually be extruded and may then be taken up easily. It may be necessary to insert the tube into the lesion or uncover it completely because of its loculation The contents of several vesicles may conveniently be collected in a single tube For shipment the capillary tube may be broken off above the level of the fluid and packed with absorbent cotton in a sterile culture tube. The package should be kept cool in transit, but refrigeration is not necessary

Dried crusts are best collected individually, by gently detaching them from the older lesions. For shipment they may also be placed in a sterile culture tube with cork or rubber stopper, not cotton, and shipped without refrigeration If crusts are employed, it is necessary to

be certain that the scabs which are secured are from variolous and not incidental, perhaps traumatic, lesions It is essential also that the virus be not inactivated by the application of an antiseptic oily lotion, the use of which is routine in many hospitals where smallpox patients are cared for. In the laboratory the fluid-bearing capillary tube or dried crust from a suspected lesion is placed in a mortar and ground thoroughly with 1 or 2 ml. of saline solution. The cloudy suspension is pipetted off, and 1:10 and 1:100 dilutions made from it. A 10- or 11-day embryo should be used Before preparing the embryo for inoculation, about 500 units of penicillin in a volume of 1 ml. should be injected into the yolk sac. For this purpose the solution is placed in a sterile syringe fitted with a 22-gauge needle. The needle is thrust into the small end of the egg, far enough to bring the needle point approximately to its center. The measured volume of fluid is expelled, the needle withdrawn, and the hole sealed with a drop of hot paraffin. For inoculation the egg is placed on its side, and an opening is made in the air sac with a pointed instrument. With a hand-grinding tool or a triangular file, a window about 1 cm. on a side is cut in the shell over the embryo, the shell removed, and the shell membrane carefully opened. This allows the chorioallantoic membrane to fall away from the shell. If it tends to adhere, gentle suction on the opening in the air sac exerted with a small rubber bulb will usually detach it easily. Approximately 0 1 ml. of the suspension of suspected material is dropped onto the surface of the chorioallantois and the window sealed with scotch tape At least 3 embryos should be inoculated with each dilution. After 3 days the egg is opened and the membrane removed and spread out in a shallow dish for inspection.

The lesions produced by vaccinia and variola (as by several other viruses) are seen as discrete, opaque areas in the delicate membrane.[4] Those due to variola tend to show more proliferation and less necrosis and are therefore larger and denser than those due to most strains of vaccinia The embryo is less likely to be killed by variola virus. With a massive infection the lesions fuse into a confluent mass. The variation in appearance of lesions produced by different strains of vaccinia is great enough, however, to require that positive differentiation between vaccinia and variola should be undertaken only by one fully acquainted with the character of the lesions. On the other hand no disease that is a papulovesicular eruption in man gives a similar lesion in the embryonic membrane so that the diagnosis "infection with variola—vaccinia virus" can be made with some assurance. For separa-

tion of variola and vaccinia the different host ranges of the viruses are utilized—vaccinia is readily transmissible to rabbits by dermal or intradermal inoculation; variola gives inconsequential if any lesions and is very difficult or impossible to maintain in series. The infected membrane should therefore be suspended in saline solution for further passage in the chick embryo and for inoculation of rabbit skin and rabbit cornea. A virus originating in man giving discrete lesions on the chorioallantoic membrane of the chick, producing keratitis in the rabbit with Guarnieri bodies in the corneal cells, and producing inconsequential or no lesions on the rabbit skin may be considered variola. A virus from man giving similar discrete lesions in the chick embryo, keratitis in the rabbit, and well-developed lesions in the rabbit skin may be considered vaccinia Final identification may be assured by preparing serial 10-fold dilutions of the chick membrane in saline solutions, covering the range 10^{-1} to 10^{-8}. Two series are prepared from these suspensions, to one of which an equal volume of normal rabbit serum is added, to the other undiluted serum from a rabbit immune to vaccinia, and each is inoculated intradermally in a rabbit. Final observations are made at 5 days, when if the virus is vaccinia a pronounced difference in titer should be apparent between virus mixed with normal and with immune serum.

The virus may be preserved for future study by dropping the infected membranes in a 50 per cent solution of glycerol in saline solution, by freezing, or by drying from the frozen state.

III IMMUNOLOGIC DIAGNOSIS

The most satisfactory immunologic methods for diagnosis of variola and vaccinia involve the use of antigens derived from the eruptive lesions Viral antigens in the lesions may be identified by the complement fixation or flocculation tests, using hyperimmune rabbit serum as the test reagent.

A COMPLEMENT FIXATION TEST

A satisfactory complement fixation test may be performed according to the following technic [6]

1. *Hemolytic system* Prepare a 4 per cent suspension of thrice-washed sheep cells in saline solution and sensitize by the addition of 2 units of antisheep amboceptor. Prepare complement from the pooled serum of 4 normal guinea pigs and titrate against the sensitized cell suspension. For the complement titration, use 0 2 ml. of sensitized cells. Adjust the total volume of the test to 0.5 ml. and determine the minimal

completely hemolytic unit of complement after incubation in the water bath at 37° C. for 30 minutes. The guinea pig serum should be so diluted *that 2 units of complement are contained in 0.1 ml.*

2. *Hyperimmune serum.* Rabbits convalescent from vaccinal infection should be hyperimmunized by the intravenous injection of bacteria-free vaccinal antigen which is conveniently prepared from rabbit testicle. The organs are removed 4 days after inoculation with passage virus, the absence of viable bacteria demonstrated by culture, and a 10 per cent suspension prepared in physiologic saline solution. Gross particles are removed by low speed centrifugation, and the supernatant fluid is stored at 5° C. until used. Rabbits are injected on 2 or 3 successive days of each week for 4 weeks with 1.0, 1.5, 2.0, and 2.5 ml. of the viral suspension A test bleeding is made about 10 days after the last injection. A satisfactory serum should fix complement in a titer of 1:32 or more Immediately before use, the serum must be heated to 56° C. for 10 minutes to remove a heat-labile, anticomplementary activity which develops in rabbit serum Dermal antigens should not be used, because of the strong probability that the resultant serum, containing antibodies against bacteria, will fix complement with material from bacteria-containing but nonvaccinal lesions

3. *Vaccinal antigen.* A standard vaccinal antigen should be prepared for titration of hyperimmune serum and for use as control material in the complement fixation test. This may conveniently be made from testicular passage virus The infected testicles are minced finely and ground without abrasive, with a small quantity of saline solution. After a few minutes' sedimentation, the supernatant fluid is removed and dried from the frozen state. The dried virus may be ground to a fine powder in a mortar, and either stored in a desiccator at 4° C or distributed in ampules which are evacuated and sealed For use a 1 per cent suspension is prepared and centrifuged at 1,500 r.p.m. for 15 minutes. The dried antigen may be kept indefinitely; the solution should be prepared fresh for each run.

4. *Test antigen.* The contents of 1 or 2 vesicles or pustules should be washed out into about 0 2 ml of saline solution, or the fluid-bearing part should be placed in a sterile mortar and ground with a similar quantity of fluid. If dried crusts have been collected, 2 or 3 should be placed in a small mortar and ground with 0 2 or 0 3 ml of saline solution. After thorough dispersion of the material, it is divided between the 2 tubes of the test.

5. *Procedure for test.* The test should be run in small culture tubes (size 10 by 75 mm. is convenient). Fixation is carried out for 4 hours at 5° C. Sensitized cells are added, and the results read after secondary incubation for 30 minutes at 37° C. in the water bath. A positive control should always be included. For this purpose a 1 per cent suspension of the dried testicular antigen is convenient, 0.05 ml. being used in the test. The procedure is summarized in Table 1.

TABLE 1

OUTLINE OF PROCEDURE FOR COMPLEMENT FIXATION TEST FOR VACCINIA AND VARIOLA

Antigen (1 vesicle in 0.1 ml)	0.1 ml.	0
Immune rabbit serum (diluted 1:10)	0.05 ml	0.05
Complement (2 units)	0.1 ml.	0.1
Saline solution	0.0	0.1
Fixation, 4 hours at 5° C.		
Sensitized sheep cells	0.2 ml.	0.2 ml
Incubation, 30 minutes at 37° C		

NOTE—A parallel test should be done, using known variolous material or 0.05 ml. of a 1 per cent testicular virus extract

B. FLOCCULATION TEST

A flocculation test for identification of the antigens of variola and vaccinia may be performed according to the following technic.

1. *Hyperimmune serum.* The specific serum is prepared as for the complement fixation test. A satisfactory serum should produce a flocculent precipitate with vaccinal antigen in a dilution of 1:32 or higher.

2. *Control antigen.* Either testicular or dermal passage virus extract may be used for titration of immune serum although the dermal extract is more satisfactory. It is prepared as follows. A rabbit is inoculated over a large area of the back with a potent vaccine virus by light scarification. On the 3d day the animal is sacrificed, the skin removed and pinned on a board, and the vaccine pulp harvested into about 20 ml. of distilled water buffered to a pH of 7.0 (McIlvaine's buffer diluted 1:50). This material is centrifuged at high speed, and the lightly opalescent supernate used in the reaction. The solution is fairly stable if stored at 4° C. with ether added as a preservative.

3. *Test antigen.* The antigen is prepared as for the complement fixation test. However, the contents of 4 or 5 vesicles, or 4 or 5 crusts, should be suspended in 0.5 ml. of fluid, the suspension transferred to a culture tube, and gross particles removed by light centrifugation. Solution of the dried vaccinal antigens is facilitated if the material is suspended first in a small quantity of 8.5 per cent solution of sodium chloride, and then 9 volumes of distilled water added to make the final concentration of 0.85 per cent.

4. *Procedure.* In performing the test, 0 2 ml. of antigen are mixed with 0 2 ml of immune serum diluted 1:10. A control tube contains 0 2 ml. of antigen and 0.2 ml. of diluted normal rabbit serum. A parallel test is run, using known vaccinal antigen. After incubation overnight at 50° C. the presence or absence of flocculation is determined, preferably with the aid of a low power hand lens.

While the complement fixation and flocculation tests do not distinguish between the viruses of variola and vaccinia, a positive result with either one suffices to establish the diagnosis of infection with one of the viruses, and differentiation between the two is usually not essential. The complement fixation test is the more sensitive, and positive results may be obtained consistently with the content of a single vesicle as antigen Interpretation of the results of this test is also usually more clear cut. The serologic tests for identification of viral antigens may be performed promptly after receipt of material, and the results are immediately available.

Tests for antibodies may be applied also to the patient's serum The diagnosis, however, in this case is not available until the patient is convalescent, and a speedy result is usually desired Further, the results may be confused by the presence of antibodies resulting from previous vaccination for smallpox

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INFLUENZA

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I. INTRODUCTION

INFLUENZA is an acute infection of the respiratory tract which usually occurs in epidemic form and is characterized by sudden onset of fever, malaise, and respiratory symptoms. There is nothing pathognomonic about its symptomatology, and it is difficult or impossible to differentiate clinically a sporadic case from many other infections of the respiratory tract that are common in winter months. In epidemic form,

however, the diagnosis is less difficult While the prostration at the onset of an attack may be sudden and severe, the disease is usually not serious, the fever subsides in 3 or 4 days, and complications are uncommon.

In 1933 it was established that influenza is a virus infection,[1] that the infective agent is present in human upper respiratory secretions during the acute phase of illness, and that an attack was followed by a sharp rise in the patient's neutralizing antibody titer.[2] In 1940 a second kind of influenza virus was discovered,[3, 4] and the two are now referred to as influenza virus, types A and B. Neutralizing antibodies prepared against one type are completely inactive against the other, and this antigenic dissimilarity is the most pronounced difference between them. Both types produce a similar disease picture but are different in their epidemiologic behavior. Sporadic infections with type B are more common than with type A, but epidemics of A occur more often (approximately every 2 to 3 years) than those of B (4 to 6 years).[5] An epidemic is caused by one or the other type but usually not both, and there is no clear-cut evidence for postulation of further types to explain the epidemic disease On the other hand, only a minority of sporadic influenzalike infections yield evidence of involvement with either type A or B.

The agents causing influenza are typical viruses of medium size (circa 80-100 mμ) and are distinguished from many other filtrable agents in the ease with which they may be handled in the laboratory, where accidental human infection has been rare and never serious. Human strains of influenza virus are capable of infecting a number of different mammals, of which the ferret is the most susceptible The virus will also infect mice, and with repeated passage becomes adapted to this animal, causing extensive pulmonary consolidation and death In both these animals, when the virus is given intranasally, the infection produced is limited to the cells of the respiratory tract, and the same is probably true of man Infection of man or other mammals stimulates antibody formation in the host so that serum taken after convalescence when mixed with active virus is capable of abolishing virus infectivity (neutralization)[2] Influenza virus also grows rapidly and profusely in several tissues of the developing chick embryo where even a high level of infection is accompanied by no grossly detectable lesions.

Nearly all strains of influenza virus are capable of agglutinating the red blood cells of certain species, most notably those of birds, the guinea pig, and man This property, which is shared with a few other human viruses, can be used as a means of detecting the presence of the agent in the developing chick embryo. The ability to use chick embryos for isolation of virus from patients has greatly facilitated the use of this procedure in the routine laboratory. The hemagglutinin of influenza virus may also be used for the titration of antibody

Since the clinical picture of influenza is not too sharply defined, the only certain method of diagnosis is through laboratory methods. These consist of (1) isolation of influenza virus from the throat washings of acutely ill patients and (2) detection of a rise in the patient's antibody titer between the acute and convalescent period. Each method has its advantages and may best be used in conjunction with the other. The isolation procedure, under ideal conditions, gives the most rapid result, which when positive is unequivocal; the serologic methods are technically simpler and give a high percentage of positive results in cases of influenza, but usually the diagnosis is resolved only after the patient is well.

II. DIAGNOSIS OF INFLUENZA BY THE ISOLATION OF VIRUS

From limited present knowledge it is clear that the isolation of influenza virus from patients can best be achieved through attempts to grow the agent in chick embryos. Other methods have been employed in the past, and they may be of value in special instances but none, except the chick embryo technic, is readily adaptable to the average bacteriologic laboratory. The chick embryo has been extensively used for this purpose only in recent years and much remains to be learned about the method.

A MATERIALS NECESSARY FOR VIRUS ISOLATION TECHNICS

For isolation procedures, the materials necessary but not ordinarily found in a bacteriologic laboratory are as follows.

Chick embryos For the two isolation technics to be described, chick embryos of 11 and 13 days' incubation are required White Leghorn eggs, because of their transparent shell, are much to be preferred, and they should be obtained from pullorum-free flocks If the preinoculation incubation is carried on in the laboratory, a commercial type of incubator is desirable in order to obtain a high yield of living embryos. If incubated eggs are purchased direct from a poultryman, the ordinary laboratory types of incubator may suffice for use after inoculation

An incubator set at 35° C for use after inoculation is helpful. This is optional equipment, but there is evidence that some strains grow better at this temperature than at 37.5° C

A drill for making windows and scotch tape for sealing them If the amniotic method of inoculation is used, and windows are to be made, some type of drill for scoring the eggshells is necessary. A hand drill with a flexible shaft and a sharp-edged carborundum disc is best for this, or a small utility tool driven by a motor in the handle will work very well. Scotch tape, one inch in width, is ideal for rapid sealing of windows, although cover slips may be used.

An egg candler. This is necessary for discarding infertile eggs and for locating the position of the embryo prior to cutting windows. A candler with a very bright light works best

Type specific antisera prepared against representative A and B strains. Suitable sera may be prepared, using rabbits, chickens, ferrets, or other animals. Horse and goat sera have been used Antigens should be prepared from allantoic fluid by the method described in Section III, C. PR8 and Lee (A and B)[6,8] are standard strains for this purpose, but others may be used. With rabbits and chickens large inocula are tolerated, and 10 ml. or more may be given at one time. Intravenous inoculation is the best, but intraperitoneal injections are also efficient. Animals should be bled for testing 2 weeks after injection, and another course of inoculations given if the titer level is not sufficient. Friedewald has found that the use of adjuvants with subcutaneous inoculation produced the most uniformly high levels.[7] Very high levels of antibody are desirable, and there should be a serum titer 1:250 to 1,500 with the homologous strain by the pattern method of inhibition titration given in Section III, E, 1, c. If ferrets are available, very good antisera may be prepared by infecting the animals by intranasal inoculation of a 10^{-2} or higher dilution of infected allantoic fluid and by bleeding out the animal in 3 weeks The use of merthiolate for preserving the sera does not interfere with their use in any way. A supply of normal serum from the animal used is also needed

B. Collection of Specimens

For the isolation of influenza virus from patients, pharyngeal washings are used. Optimally, the washing should be obtained during the first 3 days of illness and while the patient is still febrile, although virus may be recovered 5 or 6 days after onset. The patient is asked to gargle with 10 to 15 ml. of some buffered solution, such as broth containing buffers or saline containing 5 per cent of heat-inactivated horse serum It is a good idea to ask the patient to cough just before taking a washing, on the chance of bringing some infective material from the trachea into the pharynx. When possible, it is advantageous to test a washing for virus immediately. If there is a delay of a few hours, the washing should be kept chilled in ice, but if the delay is much greater, the fluid should be stored at low temperature, preferably that of dry ice. Washings may be preserved and shipped in glycerol, but this is not ideal.

C. Isolation of Virus by Allantoic Sac Inoculation

Two general methods for the use of chick embryos in isolation procedures will be described (Section II, C and D). Except for the route of inoculation and the age of the embryos used, the two methods

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An incubator set at 35° C. for use after inoculation is helpful This is optional equipment, but there is evidence that some strains grow better at this temperature than at 37.5° C

A drill for making windows and scotch tape for sealing them If the amniotic method of inoculation is used, and windows are to be made, some type of drill for scoring the eggshells is necessary A hand drill with a flexible shaft and a sharp-edged carborundum disc is best for this, or a small utility tool driven by a motor in the handle will work very well Scotch tape, one inch in width, is ideal for rapid sealing of windows, although cover slips may be used.

5. *Testing of fluids for hemagglutinins.* Several ways of testing the fluids for hemagglutinins may be used, but only one will be described in detail. As stated above, 1.0 ml of fluid from each egg is placed in a tube for testing. This should preferably be a tube of 10 mm. internal diameter or less, although larger tubes will do. Some of the fluids may contain a heavy precipitate of amorphous urates, and this may usually be redissolved by warming the tubes in a 37° water bath. The presence of a small amount of finely divided debris does not interfere with the test, but it is better to remove gross particles by slow speed centrifugation. Fluids containing yolk give results difficult to interpret.

To each tube is added 1.0 ml. of a saline suspension of washed chicken red cells. The red cells should be 1 to 1.5 per cent by volume, and the details of their preparation are given in Section III, E, 1, a. A saline control should be included. The cells are thoroughly mixed by shaking and are allowed to stand an hour or more at room temperature. Agglutination when present is usually vigorous, causing the cells to clump and settle out rapidly as compared with the control and may often be seen in 10 or 15 minutes. Very slight degrees of agglutination causing a peculiar pattern formation on the bottom of the tube should be considered tentatively negative until the material has been tested by further passage.

Further description of the appearance of agglutination is given under serologic methods. A much more delicate test for hemagglutinins, the pattern test, which has the advantage of using less allantoic fluid, may also be used and is described in Section III, E, 1, b. It is also possible to open the eggs without preliminary refrigeration, the torn membranes are allowed to bleed, and the bloody fluid is placed in a test tube. This eliminates the special preparation of red cells.

a. False positive agglutination. False positive agglutination may be due to several causes.

(1) The presence of amorphous urates often causes confusing results, since the precipitate settles and brings down red cells with it, and pattern formation on the bottom of the tubes may also occur.

(2) The presence of yolk sac material also causes similar results which are difficult to interpret.

(3) Certain bacterial contaminants may be present which grow profusely in the allantoic fluid and cause definite clumping of red cells.

Allantoic fluids should be routinely plated on agar to detect gross contamination by bacteria. One point on which the false types of agglutination may be differentiated from that due to virus is in titer. Virus agglutinins will usually give a detectable effect in a dilution of 1:16 or greater, whereas that due to bacteria or precipitates will not often go beyond 1:4 and usually less.

are similar in principle. In the first, the throat washing is inoculated into the allantoic sac, and in the second, material is put into the amniotic sac. The allantoic method was first successfully employed by Rickard, Thigpen, and Crowley [8] and is exceedingly simple from a technical standpoint. A comparison of both methods is given in Section II, E.

1. *Preparation of eggs.* For this method embryos which have been incubated for 10 or 11 days are used. The eggs should be placed on their sides in trays convenient for handling. The uppermost side is swabbed with 70 per cent alcohol or other disinfectant. A blunt dissecting needle with a heavy handle is a good instrument for puncturing the shells The air sac end of the egg is first punctured, through the eggshell membrane but not into the egg proper. Next a hole is made on the upper side of the egg toward the air sac end, being careful to go through the shell but not through the eggshell membrane. The needle should be flamed between punctures. The hole is sterilized with alcohol.

2. *Preparation of inoculum* The inoculum is prepared by mixing unfiltered and uncentrifuged throat washing with sufficient penicillin for a concentration of 250 to 1,000 units per ml.

3. *Inoculation of eggs.* Ordinary 1 ml tuberculin syringes are convenient for inoculation and should be fitted with a 23-gauge, or smaller, needle of about one-half inch in length The point of the needle is inserted obliquely in the hole in the side of the egg so that the beveled tip is just out of sight and 0 2 ml. of washing inoculated. The hole is covered with warm melted paraffin. Six or more eggs per washing *should be used The eggs are incubated for about 48 hours at either* 35° or 37° C, preferably the former.

4. *Harvesting of allantoic fluid* For harvesting the allantoic fluid without contamination with embryonic blood, the eggs should be placed for several hours, or, better still, overnight in a refrigerator. To aspirate the fluid the egg is placed in holder, and the shell over the air sac is picked away with forceps The eggshell membrane and the chorioallantoic membrane underlying it are then torn back with blunt forceps, and the allantoic fluid is removed. A 5 ml pipette with a rubber bulb is most convenient, and the embryo and membranes are depressed with the forceps to keep them from interfering. As a routine 1.0 ml of the aspirated fluid is put in a tube suitable for the agglutination test, and the rest is pooled with that of other eggs for further passage if such is necessary.

D ISOLATION OF VIRUS BY AMNIOTIC SAC INOCULATION

It has been known since 1940[8] that influenza virus from human sources would grow well when material was inoculated into the amniotic sac. The method was not very useful, however, until the red cell technic was used as a means of identifying the infection. Since 1942 the method has been extensively tested by several investigators, with evidence that it is the most sensitive method for detection of virus in human washings. Of the several possible methods of amniotic inoculation, the window technic, developed by Burnet[9, 10] will be described first and in greatest detail, with certain modifications. While this is the most time-consuming method, it is also the surest means of proper inoculation.

1. *Preparation of eggs.* Eggs of 13 days' incubation are essential for the success of this method, since the results are not nearly so regular with younger embryos. The egg should be candled with the embryo uppermost, and the area over the embryo marked. With a drill a circular area of about 1.5 cm. in diameter is lightly scored over the marked area Near the air sac end, a small wedge-shaped sector is cut in the circle, around which the eggshell is carefully cut through to the eggshell membrane with care to avoid hemorrhage This should be done shortly before inoculation, and the shell wiped with 70 per cent alcohol The small triangular piece of shell is first removed with a stiff needle or fine forceps, being careful not to puncture the underlying membrane. A hole is punched through the shell over the air sac. A drop of saline is placed on the exposed eggshell membrane, and a hole is teased in this membrane with a sharp needle, without puncturing the chorioallantois By applying gentle suction with a rubber nipple over the air sac hole, the chorioallantoic membrane will drop, and this may be seen through the eggshell membrane The saline reduces hemorrhage from this procedure. After the membrane has dropped, the rest of the scored circle may be picked away with forceps.

2 *Preparation of inoculum.* The inoculum is prepared by mixing untreated throat washing with sufficient penicillin to bring the concentration to 250 to 1,000 units per ml Two- or three-tenths of an ml. of inoculum is taken into a 1 ml tuberculin syringe, with a half-inch, 24 to 26 gauge needle, and 0.1 ml. of air is also aspirated.

3. *Inoculation into the amniotic sac.* The amniotic sac surrounds the embryo and underlies the chorioallantoic membrane The amniotic

6. *Further treatment of negative material.* If the results of hemagglutination tests are negative or questionable, it is sometimes profitable to make one or two more passages by the allantoic route. For this the fluids of the first passage are pooled and inoculated into the allantoic sac of a group of 11-day embryos, which are then treated the same as those of initial passages. It is advisable to add penicillin to the passage fluid. Little is to be gained by going beyond three negative passages.

7. *Typing of strains.* The presence of potent chicken cell hemagglutinins in allantoic fluid after inoculation of throat washings is in itself good evidence of the presence of influenza virus, but the further supportive proof of differentiation as to type should be routine. This may most conveniently be done by agglutination inhibition tests with type specific animal antisera. The full description of this technic is given under Section III, E, 1, c.

The hemagglutinin must be titered (usually by the pattern method since the quantities of fluid are limited) and 4 to 8 units of hemagglutinin are then tested with dilutions of antisera of types A and B and *with a normal serum* from the same animal. Specific inhibition by either antiserum indicates the virus type

The typing of newly isolated strains of influenza virus is often not easy, due principally to the fact that high dilutions of normal serum, especially rabbit, possess the power to inhibit the hemagglutinin in the same manner as antibody so that with a new A strain, for example, the A antiserum and the normal serum may inhibit agglutination at nearly the same dilution This is especially true when the strain used for preparing the antiserum and the new strain are antigenically quite different This difficulty in strain typing may be circumvented in a number of ways

a By the use of sera which possess very high levels of antibody so that the specific inhibition of agglutination will occur well out of the normal inhibitory zone.

b By the use of antisera prepared against several antigenically different strains of the two types of virus The serum against the most closely related strain will usually give the best result

c By the passage of the new virus by the allantoic route for a few times which often reduces the inhibitory titer of normal serum for the strain, making it more readily typable

d By the *in ovo* neutralization test, described briefly in Section III, H, 2, since the agglutinin inhibitory factor in normal serum does not usually *neutralize* the virus.

e. By the use of paired human sera Since the normal inhibitory factor in human sera seems to be less prominent than in certain animal sera this method may be successful where others are not. Two pairs of human sera are used, one known to have a large difference in titer for A and the other for B strains. The unknown strain is set up for inhibition tests with all four sera, and the unknown strain should show a rise in antibody titer with only one of the pairs.

to the routine described under the allantoic technic (Section II, C, 5). Since the amount of amniotic fluid is often small, it is usually best to use the pattern method for detection of hemagglutinins (Section III, E, 1, c). Something may be gained by testing the fluids with both chicken and guinea pig red cells, although very few positives will be missed with only the former.

Since amniotic fluid is very viscous and frequently contains debris, it often gives false positive agglutination patterns (especially with guinea pig cells) when used in low dilutions. It is advisable to dilute the fluid 4 or 8 times for the agglutination test. This saves fluid, eliminates false positives, and no true positives are missed. Frequently, it is convenient to test the fluids in a series of dilutions so that the titer may be estimated, and this facilitates further identification. Positive fluids usually have a high agglutinin titer, and where the titer is low or the patterns are questionable they should be called negative, pending further passage.

6. *Further treatment of negative passages.* If the fluids of an initial passage are negative or questionable in all fluids, and especially if influenza B is suspected, a further passage is justified. Amniotic fluid may be passed by the amniotic route (undiluted) or better still the lungs and tracheas of the first passage embryos should be dissected out, ground in a mortar with sand, diluted with amniotic fluid of the same passage, and inoculated into the amniotic sac of 13-day-old embryos. More than two passages is not profitable.

7. *Treatment of hemagglutinin positive fluids.* Positive results should be checked by (*a*) serologic identification and (*b*) subinoculation by the allantoic or amniotic routes. The identification with sera has been described in Section II, C, 7. The same methods apply here, and inhibition tests should be carried out with the pattern test. By this means serologic typing may be done with an ml. or less of fluid, since the agglutinin titer is often very high. Subinoculation for another passage is not essential but will confirm any question regarding the initial results and will furnish further material for testing. Usually influenza A strains pass by the allantoic route without difficulty, but influenza B strains may fail to grow detectably in the allantoic sac.[12] In the latter case subinoculation by the amniotic route is indicated. The difficulties mentioned in Section II, C, 7 on serologic typing also apply here.

8. *Alternate methods of amniotic inoculation.* The inoculation method may be modified in several ways.

a. It is possible successfully to inject material into the amniotic sac "blindly"

membrane must be pulled out through a hole in the chorioallantois in order to gain entrance to this cavity. The simplest way to do this is with the use of fine, sharply pointed forceps. They are easily forced through an avascular area of the chorioallantois, to pick up the amniotic membrane and to pull it out. The latter is relatively avascular and has a typical appearance Being elastic, it pulls out (through the chorioallantois) into a long nipple-like protrusion, which is narrow at the base. The needle is inserted at the base of the nipple and 0.2 to 0 3 ml. of washing is inoculated. If the air in the syringe is also injected, the path of the bubbles formed will indicate whether or not the injection is into the proper cavity. Air injected into the amnion stays localized or rises to the base of the nipple that has been pulled out; if by mistake the inoculation was made into the chorioallantoic sac, the bubbles float off to the side. After inoculation the window may be closed with scotch tape.

At least 6 eggs should be inoculated with each throat washing to increase the probability of success. The eggs should be incubated on their side at 35° C This temperature is somewhat more favorable for virus multiplication than 37.5°, but the difference is not crucial.

4. *Harvesting of amniotic fluid and embryos.* Positive results may be obtained as early as 48 hours after inoculation, but as a rule more fluids will be positive after 4 days, and with influenza B [11] Burnet has found 5 days to be preferable The eggshell above the dropped chorioallantois is cut away with small scissors or may be picked off with forceps The allantoic membrane is torn off and pulled back over the edge of the shell with care, in order not to touch the amniotic membrane The allantoic fluid is aspirated or poured off It is not worth while to test it for hemagglutinins The amniotic sac is picked up with forceps If a generous amount of the sac is grasped by the instrument, the tissue is less likely to tear. A small hole is punctured through the sac with a sharp instrument, and the fluid contents are aspirated with a pipette and rubber nipple This often requires patience and ingenuity since the volume of fluid is often small, and it may be pocketed below the embryo Pipettes made of glass tubing with a wide tip work best since they are not so likely to plug Sometimes no fluid is present, in which case the sac should be washed out with 1.0 ml. of buffer or broth If further passage of the material is contemplated, the embryos should be saved.

5. *Testing of fluids for hemagglutinins* This is essentially similar

to the routine described under the allantoic technic (Section II, C, 5). Since the amount of amniotic fluid is often small, it is usually best to use the pattern method for detection of hemagglutinins (Section III, E, 1, c). Something may be gained by testing the fluids with both chicken and guinea pig red cells, although very few positives will be missed with only the former.

Since amniotic fluid is very viscous and frequently contains debris, it often gives false positive agglutination patterns (especially with guinea pig cells) when used in low dilutions. It is advisable to dilute the fluid 4 or 8 times for the agglutination test. This saves fluid, eliminates false positives, and no true positives are missed. Frequently, it is convenient to test the fluids in a series of dilutions so that the titer may be estimated, and this facilitates further identification. Positive fluids usually have a high agglutinin titer, and where the titer is low or the patterns are questionable they should be called negative, pending further passage.

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8. *Alternate methods of amniotic inoculation.* The inoculation method may be modified in several ways.

a. It is possible successfully to inject material into the amniotic sac "blindly"

without making a window. Although this technic has been successfully used in isolating influenza virus[12] and has been used by Eaton for atypical pneumonia material,[14] it is less precise than the window method

b. Taylor[15] has described a technic in which the opening is made over the air sac, the eggshell membrane removed, and the amniotic membrane pulled out through a hole in the chorioallantois as in the window method. The end of the egg is sealed with glass or plastic caps This method produces less trauma than by dropping the membrane.

E. COMPARISON OF THE ALLANTOIC AND AMNIOTIC METHODS

1. *Allantoic method simpler* The allantoic method is technically very much simpler than the amniotic method The former may be easily carried out by persons who have had little prior experience with chick embryos, and much more material may be tested in a short time On the other hand the amniotic method can be learned easily with practice but requires more competent personnel, and takes more time.

2. *Speed of obtaining results* There probably is no difference in this Positive isolations may be obtained in 48 hours by both methods, and those washings which require 96 hours to grow out in the amnion in the main would not be detected by the other method

3 *Sensitivity in detecting virus* It has been shown that the amniotic method is much the most sensitive medium for the virus from human sources,[13, 16] and more recent work shows that with influenza A this method will detect 100 to 10,000 times less virus than the allantoic technic[12a] Attempts at isolating influenza B by the allantoic route have been quite unsuccessful[12a] in some laboratories but successful in others[12b] So far the amniotic method is the most sensitive available

4 *Success with throat washings from epidemic material* The exact percentage of success in isolating virus by different means cannot be compared readily from the data of different investigators This is in part due to the variations in selection of cases for study In some series of epidemic cases the amniotic method was successful in as high as 75 per cent of the washings, when the virus was type A[10] and 30 per cent with type B[12a] Direct comparison with the same washings by the allantoic route gave much less success with both types, 20 per cent for influenza A and no success at all with B

F. SOURCES OF FAILURE AND SPECIAL PRECAUTIONS

1 Both methods may fail to yield virus from serologically proved positive cases The exact percentage of failure is not known but is greatest with the allantoic method, especially when dealing with influenza B Factors which will improve the result are:

a Careful selection of material from early acute cases of typical symptomatology.

b Inoculation of washings into embryos shortly after obtaining them (preferably before freezing).

c Storage on dry ice if there is to be a delay in culturing them.

2 Penicillin-resistant bacteria may grow out and kill all the embryos The use of sulfadiazine or streptomycin[15, 16] may overcome the interference from such infections If this fails filtration may be attempted

3 Extreme caution regarding accidental contamination of eggs with influenza virus is essential to eliminate false positive isolations[16] Since influenza virus is *relatively innocuous to human beings* and is often used in an active state for *in vitro* tests in the laboratory, it is not uncommon for active dried virus to be spread about. Since very little virus is required to infect an egg, it is essential to use every precaution against inadvertently introducing the agent into experimental embryos. This should be done by usual procedures of care in spilling active virus, by cleaning the eggs with disinfectants and inoculating them in a "sterile" room or other isolated quarters, by disinfecting the bench and hands before inoculation, and by using measures for control of dust on the floors. Even with all these precautions contamination may occasionally occur, and the danger is especially present with repeated passage of egg material Passage material should be exposed to room air as little as possible In this the amniotic method has an advantage since passage is often unnecessary.

4. *Interpretation of results.* It is obvious that a negative result cannot be interpreted as ruling out influenza, and this is especially true of the allantoic method A positive result (barring accidental contamination, which should be rare) is unequivocal since so far no convincing demonstration of virus in normal individuals (except with inapparent infections) has been made.

G. OTHER METHODS FOR DETECTION OF INFLUENZA VIRUS IN HUMAN WASHINGS

There are a number of other methods by which influenza virus may be detected in human throat washings, none of which have any distinct advantage over the methods outlined here, but some may be of service in special circumstances For details of these methods the sources cited should be consulted.

1 *Detection of virus, using ferrets* The ferret was the first experimental animal successfully employed for detection of virus from human sources[5, 19] and provides the most sensitive means of detecting the agent in mammals Untreated throat washings may be inoculated intranasally, and the temperature and clinical course followed Some strains produce a fever (occasionally diphasic), and the animal may become ill with listlessness and nasal discharge Recovery almost always occurs after a few days. Many strains, however, do not cause any detectable clinical signs, but a rise in antibody titer which follows inoculation of active material is a highly sensitive means of detection of virus infection[19, 20] Unfortunately, the ferret is an expensive animal, and its use for influenza diagnosis requires special isolation quarters to prevent cross-infection

2 *Use of hamsters for influenza diagnosis.* Methods similar to those de-

scribed for ferrets have been applied to the use of hamsters.[20] Although this animal is smaller, cheaper, and easier to house than the ferret, it also is not quite so sensitive as the latter to infection from human material.

3. *Primary virus isolation in mice.* Some strains of influenza virus may be adapted to mice directly from throat washings,[21] and the inapparent infection on initial mouse passage may be followed by the development of immunity to laboratory strains.[22] The method has not been widely employed

4. *Detection of hemagglutinins in throat washings.* Attempts have been made to detect hemagglutinins in throat washings, and while specific agglutination, inhibited by specific sera, has been described,[23] the results seem somewhat obscured by the fact that a great many washings show agglutination which is nonspecific.

5. *Concentration of virus in throat washings.* Methods of concentration of virus from throat washings have also been attempted, but so far no technic has been described which will work on the high dilutions of virus usually encountered

III. SEROLOGIC DIAGNOSIS OF INFLUENZA

A. GENERAL CONSIDERATIONS

The serologic method depends on the occurrence of antibody production in human beings following influenzal infection. Almost all individuals (except in early childhood) have circulating antibodies for influenza A and B at the onset of an infection, and the circulating titer may be quite high. Between 6 and 10 days after the onset of an infection, the antibody titer for the type of influenza virus causing the illness begins to rise, reaching a maximum in two or three weeks, after which the level begins to decline. This rise in titer is the basis of all serologic diagnostic tests.

There are many available strains of both types A and B, and within each type all the strains have some degree of antigenic relationship, though some are very dissimilar. In general, the antibody response of a patient to an A strain may be measured by many other A strains, but the degree of response varies. Since the antibody response in most adults is by no means clearly strain specific, it is impossible to predict with accuracy what the best test strain will be. Though a strain from the outbreak in question is often the most sensitive in detecting antibody rises in actual practice, the PR8 strain of influenza A [3] and the Lee strain of influenza B [4] are the two most commonly used, and they give satisfactory results.

The rise in antibody titer may be measured on paired sera in several ways; neutralization tests in mice or chick embryos or by *in vitro* methods of complement fixation or agglutination inhibition. There is no wide disparity in the results with any of these methods, and for

practical purposes it seems that all of them measure the same type of antibody. The antibody level of an individual who has not been recently infected remains fairly constant or drops very slowly over long periods of time. The size of the critical or diagnostic rise depends, therefore, on the inherent errors in the methods used for testing.

Since the antibody titers of normal sera cover a wide range, including very high levels, the testing of a single convalescent serum is of no diagnostic value. It is absolutely necessary for serologic diagnosis of influenza in the individual case to have acute and convalescent phase serum specimens on which to make comparisons.

B Collection of Sera

For diagnostic purposes it is best to have the acute phase serum taken very early in the disease, and certainly not later than the 5th day. A rise in titer may occur as early as the 8th day, but the convalescent specimen should be taken from the 10th to the 14th day after onset, though a diagnosis may be made with second specimens taken after a month or more The serum should be clear and free of hemolysis.

C Preparation of Virus

The virus preparations needed are essentially the same for any of the *in vitro* methods to be described and may also be used for the neutralization test The strains chosen would be cultured in the allantoic sac of 11-day-old chick embryos If the seed virus is in allantoic fluid it should be diluted to 10^{-3} to 10^{-6} before injection The material may be diluted in broth or other buffered solution. The inoculation is done as described in Section II, C, 1 and 3. The embryos are incubated for 48 hours at 37° C, chilled in a refrigerator overnight, and the allantoic fluid harvested as described in Section II, C, 4. The fluids of all eggs are pooled, and the pool is tested for hemagglutinin titer (Section III, E, 2, c) To be satisfactory as antigens the hemagglutinin titer should be fairly high (1 500 or more by the pattern method of titration), and if it is less than 1:4 (initial dilution) it is not suitable for use If the titer of a pool is not satisfactorily high, either a different dilution of inoculum or else new passage material should be tried In this regard it should be emphasized that increasing the concentration of inoculum will not necessarily give better results and may lessen the amount of virus harvested This is probably because of interference effects from inactive virus in the inoculum,[31] and for this reason

seed material should be preserved in a way to do the least damage to active virus.

The pool of allantoic fluid should be tested for sterility and centrifuged for 10 or 15 minutes at 1,000 to 1,500 r.p m. to remove the cellular debris and blood.

1. *Methods of storing virus.* Generally it is most convenient to make a large amount of virus at one time and store it for future use. This may be done in several ways:

a Pooled allantoic fluid as it comes from the eggs may be stored in a refrigerator at 4° C. for periods up to several weeks without loss in complement fixing or agglutinin titer. After a variable period of time, however, a precipitate forms which will not readily go back into solution on warming. This precipitate adsorbs the virus in suspension and markedly reduces the titer

b. If the titer of hemagglutinins in allantoic fluid is much higher than that needed for serologic testing, the fluid may be diluted with buffer or saline to the concentration (or slightly greater) suitable for use in serologic tests. This dilution will usually prevent or greatly delay the formation of a precipitate, and the hemagglutinin titer may remain constant for months

c. The allantoic fluid pools may be kept in a frozen state at -20° C in a mechanical refrigerator or at -70° C in a CO_2 ice box In this form the titer of hemagglutinins will be preserved indefinitely, and while a precipitate forms on thawing, this will generally go back into solution at 37° C.

d. Allantoic fluid may be dried on a lyophile apparatus after which its titer will remain constant over a long period. This is convenient for tests in the field

e The undesirable solutes in allantoic fluid may be eliminated by dialysis of the virus suspension through cellophane against saline or buffer, and then kept in a fluid state in a refrigerator.

f. The virus may be concentrated by centrifugation at high speed (10,000 r.p m or more in an angle head for 1 hour) and resuspended in buffer or saline. The concentrated virus is quite stable over long periods and retains its infectivity

If for any reason it is impracticable to produce virus for diagnostic purposes in the laboratory, it may be possible to obtain suitable antigens from commercial houses engaged in the manufacture of influenza vaccine It is necessary, of course, to obtain the A and B antigens separately

D. THE COMPLEMENT FIXATION TEST

Complement fixation[25] has been widely used as a test for antibodies in influenza with excellent results, though it is perhaps less widely used than methods employing the red cell agglutination principle. The test may be employed with a number of variations of detail, none of which depart widely from the methods used for complement

fixation in other systems. No detailed description will be given here, and other texts on the complement fixation technic should be consulted by those not familiar with the general method.

Allantoic fluid preparations of virus make excellent complement-fixing antigens, and the antigen titer is in general proportional to the hemagglutinin titer.[26] With high titer preparations the antigen may be diluted 10-fold or more for the test; with poor preparations it may have to be used undiluted. The necessary antigen dilution may be gauged with a little experience from the hemagglutinin titer.

Complement should be employed at a concentration of two 50 per cent hemolytic units. The complement should be titrated in the presence of the antigen at the dilution to be used in the test, since allantoic fluid contains substances which enhance the titer of the complement present.[27] With the use of more complement, many low end points are missed, whereas less complement introduces too many anticomplementary effects.

The human sera should be tested in pairs and prepared in series of 2-fold dilutions, after heat inactivation at 56° C for 30 minutes. Six to 8 dilutions for each specimen is usually sufficient to cover the necessary range, and the optimal initial dilution depends on the strain being used. It usually saves time to test sera against types A and B at one time and, of course, necessary controls for anticomplementary effects and a known positive serum should be included. Ordinarily, serum complement and antigen are incubated for 1 hour at 37° C. before adding the sensitized cells. The test may be read after further incubation of one-half hour or after standing overnight in a refrigerator.

In general only 4-fold or greater rise in titer from the acute to the convalescent stage is considered significant of infection. Under some circumstances a repeatable 2-fold rise may have meaning, especially if a photometric method of measurement is used. Friedewald[27] has described the details of such a method which has the advantage of improving the accuracy and objectivity of the end points obtained.

The difficulties encountered with complement fixation are the same as those found elsewhere. Occasional sera are too anticomplementary to determine the antibody titer. In other sera the end points are too low to give fixation in the initial serum dilution. The general implications and limitations of serologic methods are given in Section III, F.

E. SEROLOGIC METHODS EMPLOYING RED CELL AGGLUTINATION

The uniform ability of influenza strains to agglutinate red cells has furnished another basis for measuring antibodies since the virus when mixed with sufficient antibody is no longer capable of agglutinating red cells, a reaction hereafter referred to as inhibition This method of antibody titration gives results which correlate well with those of *in vivo* neutralization studies and has been found by many workers to be simpler to perform than complement fixation. There are two general ways of demonstrating agglutination and its inhibition.

1. *The pattern method.* This method is by far the easiest technic of performing agglutination tests and is most suited for use in a general laboratory since it requires no apparatus not generally readily available and requires only very small quantities of reagents The method has been used by a number of investigators, but the details of a precise technic have been most clearly set forth by Salk,[28] to whom the reader should refer for further details The technic depends on the fact that red cells alone when suspended in a Wassermann tube settle down in a small, sharply outlined button on the bottom of the tube whereas if sufficient influenza virus is present the settling cells form a diffuse disc over the entire bottom of the tube, sometimes with characteristically serrated edges of quite typical appearance (Figs 1 and 2).

a Reagents and materials

(1) Red cells The most satisfactory cells are the erythrocytes of domestic fowl, which may be obtained by bleeding from a wing vein or by collecting in an abbatoir. The blood is citrated, filtered through gauze to remove large clots, and then washed several times in physiologic saline to remove plasma elements After the first sedimentation the buffy coat should be removed, and in the final washing the cells should be centrifuged for 10 minutes at 1,200 to 1,500 r p m They are stored for future use in a packed state at 4° C and this constitutes 100 per cent cells If sterile, the cells may be used for 1 or 2 weeks. When it is not convenient to use avian cells, human erythrocytes of the O type may be substituted and should be treated in the same manner

(2) The red cell suspension for use in this test is 0 25 per cent in strength (0 25 ml of packed red cells per 100 ml of saline) Some prefer a stronger suspension

(3) Virus suspensions are made in the way described in Section III, C

(4) Wassermann size tubes work well, but almost any similar type of test tube may be adapted to use

(5) A mirror assists in reading tests but is not necessary

b. Preliminary titration of virus. Before testing any sera it is necessary to know the strength of hemagglutinin in the virus suspen-

sions. Since this titer varies (a total of about 2-fold) with different lots of red cells, it is advisable to test the agglutinin titer on the day of the serologic test. An alternative of this is to use a small excess of virus (8 instead of 4 units) based on previous titrations.*

Twofold dilutions of virus are made in test tubes, leaving 0.25 ml. of dilution per tube. To each dilution is added 0.25 ml. of saline, which is mixed by shaking Finally 0.5 ml. of a 0.25 per cent red cell suspension is added, and after brief vigorous shaking the tubes stand at room temperature or in a refrigerator for 90 to 120 minutes or until the control cells have settled out. Without disturbing the individual tubes, the titration is read by examining the pattern of cells formed on the bottom. This is aided by the use of a mirror. Individual tubes are graded as positive or negative, and little is gained by attempts to make distinctions of intermediate degrees of agglutination. Usually the end points are sharp, going from a large diffuse disc in one tube to a completely negative, sharply outlined button in the next (*see* Fig 1), and when partial agglutination does occur it should be declared negative unless the pattern is quite typical. This involves some judgment, and subjective standards should be assumed by the operator. The initial dilution of virus suspension in the last positive tube is considered to be the titer of the suspension, and this dilution is assumed to contain one agglutinating unit. Good preparations will titer 1:200 to 1:500 by this method, PR8 tending toward the upper and Lee preparations tending toward the lower of these values.

c. Serologic test

(1) All sera must be inactivated at 56° C. for 30 minutes. Acute and convalescent pairs must be tested at the same time, and it is most convenient to test them against both influenza A and B by making serum dilutions in duplicate.

(2) Twofold dilutions of serum, 0 25 ml per dilution are made, using the same type of test tubes employed for the hemagglutinin titration. The usual initial serum dilution is 1·8, since inhibition at higher concentration is likely to be due to the normal serum factor

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*T [illegible]

(3) The virus suspensions to be used should be diluted with saline so that they will contain 4 agglutinating units per 0.25 ml. Thus, if a preliminary hemagglutinin titer was 1:64, the suspension should be diluted ¼ as much or 1:16. The virus is added to the serum dilutions, 0.25 ml. per tube. The tubes are shaken for thorough mixing.

(4) 0 5 ml. of a 0.25 per cent red cell suspension is added (*see* Section III, E, 1, a, (1)) to each tube and the mixture shaken thoroughly. The test may stand at room temperature or in the refrigerator for 90 minutes before reading.

(5) The tests are read in the same manner as the hemagglutinin titration, and the end points again are usually sharp (Fig. 2). The titer of the serum is considered to be the dilution of serum in the last negative tube.

d Special precautions and variables in the pattern type test. General remarks concerning the interpretation of serologic tests will be reserved for a later section (III, F), but some of the considerations may apply to either type of test used.

(1) By the pattern method the serologic end points are usually sharp and unequivocal, especially when Lee and PR8 strains are used for testing With some other strains the end points may be more confusing, and all strains are not ideal for this technic.

(2) To be certainly valid, a diagnostic rise in antibody titer between acute and convalescent specimens should be 4-fold or greater in magnitude Some authors feel that a rise of 2-fold by this technic is significant, but so far no decisive data have been published to establish this point From general considerations of the manner in which the test is read, it seems doubtful that a 2-fold rise has very high validity in a diagnostic sense

(3) High room temperatures should be avoided since they make the outcome of tests more variable, and in summer months especially it is best to allow the cells to settle in a refrigerator. A water bath maintained at 17 to 20° C. can be used

(4) Human O type cells may be used in place of avian cells although their use makes slight changes in technic necessary The human cells settle more slowly, and one must wait longer (120 minutes) for the cells to settle out before reading the tests There is also a tendency for cells in higher serum concentration to settle out rapidly and agglutination in this zone to disappear by 2 hours To circumvent this, Whitman [*] advocates a preliminary reading of the test at 45 minutes, at which time special attention is paid to the higher serum dilutions Whitman also advocates the use of cell suspensions of 1 per cent concentration

(5) The incubation period before reading of tests may be varied over quite a wide interval without marked alteration of the results If the tests stand for more than 2 hours, however, many of the patterns formed begin to disappear so that for maximum clarity the tests should be read as soon as the cells have settled out in control tubes.

(6) The number of units of hemagglutinin used in serologic tests may be increased somewhat without greatly changing the results of testing acute-convalescent pairs The use of 4 units has become fairly general, and the use of less than this is not advisable, but if the titer of the virus suspension is in doubt, one should err in the direction of using more than 4 units. Doubling the virus concentration will in general halve the serologic titer found without altering the percentage difference in titer between two specimens.

2. *The densitometric method.* This method employs an indirect means of measuring agglutination in which the density of cells not agglutinated is measured on some type of photometer. The transverse density of a column of normal red cells in a test tube changes very little with time as the cells settle out. When influenza virus is mixed with the cells, however, clumps are formed which settle out much more rapidly than individual cells, and there is a clearing of the red cell column, which is measured photometrically.

The main advantages of the test over the pattern technic (Section E, 1) is the increased accuracy of end point determination, which permits a serologic diagnosis to be made on the basis of a 2-fold rise in titer. It also permits more objective reading of tests Its main disadvantages are the need for certain special equipment (some type of photometer) and the increased care in making dilutions, etc, necessary to extract the full value of increased accuracy. The method has been described in detail by Hirst and Pickels,[30] including the construction of an efficient densitometer for serologic work. Miller and Stanley [31] have also described a photometric method which does not differ in any essential detail

a Equipment necessary.

(1) A photometer or densitometer Many different types of instrument capable of measuring light transmission in test tubes may be adapted to this purpose It is usually necessary to construct a simple adapter which will hold the small type of test tube used The tube holder needs to be masked so that light is transmitted through 1 cm of the length of the tube with the lower margin 5 mm from the bottom.

(2) Test tubes of 10 to 12 mm outside diameter are best for this type of test

(3) The virus used is the same as that previously described (Section III, C)

(4) Red cells are prepared in the same manner as previously described, 1 5 per cent suspensions in saline are used in this method, and their photometric standardization will be detailed below

(5) A mechanical pipetting machine either of the hand-operated syringe type (B-D Cornwall syringe) or motor-driven type is not essential, but is of very great assistance in adding reagents Red cells can, for example, be added with such force that further mixing is unnecessary.

b. Calibration of the densitometer. The red cell suspension for this type of test is made up at 1.5 per cent for a final concentration in the test of 0.75 per cent. The concentration of cells at the end point of all titrations is 50 per cent of this, or 0.37 per cent. Cells at 0.37 per cent concentration should be put in the densitometer to determine the reading at the 50 per cent sedimentation point The sensitivity of the instrument should be such that there is a good range on the scale between cells of 0.1 to 0.75 per cent, and it is best if the 50 per cent point falls near midway on this range.

Red cell suspensions prepared volumetrically at the same concentration will not usually have identical densities on an optical instrument. One should, therefore, either adjust the cells by trial and error to give a predetermined value on the densitometer or else the reading of each lot at 50 per cent of final concentration should be determined and this figure used for the 50 per cent end point.

c. Preliminary hemagglutinin titration. The setting up of both hemagglutinin and antibody titrations by the densitometric method is fundamentally the same as with the pattern technic. The basic ratio of 1 part virus, 1 part serum, and 2 parts cells is maintained throughout, substituting saline for serum in the hemagglutinin titration. The final volume is always 2 ml.

(1) Dilutions of virus in 2-fold steps are made, leaving 0.5 ml per dilution. It is better to make the dilutions in a separate set of tubes in greater volume than needed and then to transfer 0.5 ml. to the proper tube. The errors introduced by blowing out pipettes and making dilutions in small volumes may thus be avoided.

(2) Saline is added to each tube in 0 5 ml volume. The two ingredients should be mixed (As with the pattern test it is simpler to omit this step and make the virus dilutions in a volume of 1 ml., which necessitates a change in method of calculating the dilution containing 4 units in 0.5 ml.)

(3) The standardized 1 5 per cent red cell suspension is added in 1 ml. volume It is at this point that the use of an automatic pipetting machine greatly facilitates mixing

(4) The test should stand at room temperature for 75 minutes If the room temperature is very high, the tests may be placed in a large bath filled with water at 20° C. With a large volume of water the temperature will not rise very fast.

(5) The test should be read in a densitometer at exactly 75

minutes. All that is needed for calculation is the densitometric reading of two consecutive tubes, the density of which falls on either side of the 50 per cent end point. The two significant tubes can usually be determined by inspection.

(6) From the two readings the end point is interpolated by the proportional distance of the 50 per cent reading from the two figures obtained, when the dilution scale is plotted logarithmically. A very simple method for doing this with examples is included in the appendix. The virus hemagglutinin titers are roughly $\frac{1}{10}$ those found by the pattern method. The day-to-day variation in titer is about 2-fold.

d. Serologic test. From the preliminary hemagglutinin titration (c. above) the amount of virus necessary for 4 units in the serum test is calculated. For example, if the agglutinin titer was 1:57, the allantoic fluid antigen should be diluted 1:14.2.

(1) Twofold serum dilutions are made with 0.5 ml. per dilution. The serum should have been inactivated at 56° C. The initial serum dilution need not be less than 1:8.

(2) To the serum dilutions are added 0.5 ml of previously diluted virus, and this should be thoroughly mixed.

(3) The 1.5 per cent red cells are added in a volume of 1 ml. They should be freshly made up from concentrated stock just before the test. Red cells made up in the morning for preliminary titrations often change considerably in agglutinability if allowed to stand at room temperature for a few hours.

(4) The tests are incubated at room temperature for exactly 75 minutes.

(5) The end points are determined in a densitometer, obtaining two values in consecutive tubes on either side of the end point. Interpolation in terms of serum titer is essentially the same as with the hemagglutinin titration (*see* appendix).

e Special precautions and errors When carefully done this method will yield end points which are in error less than — ± 10 per cent in comparative tests run at the same time For some types of work this precision may be important In the performance of serologic tests it has the decided advantage that 2-fold differences in titer are usually diagnostically significant In one large series of tests[23] with influenza B by this method, only a little over 1 per cent of 200 serum pairs showed differences of 2-fold, and even these may have been a true antibody rise The ability to use a 2-fold limit for diagnosis greatly decreases the number of false negatives found

The variation in titer of a given serum from day to day in different tests, even with carefully controlled conditions, is about 2-fold For this reason levels

obtained in one test may not be strictly compared to those achieved on another day unless some further control is used. This is accomplished by the use of a standard serum in each test An arbitrary average value is assumed for this serum, and all titers should be corrected each day in accord with the shift in standard serum titer Thus, if the arbitrary standard serum titer is 500 and in a particular run a value of 750 is found, then all titers in this run should be multiplied by 66.

3. *Other methods of performing inhibition tests* Salk has developed a method of using blood from the finger for serum antibody titrations which obviates the necessity for venipuncture and serum separation. The technic is especially applicable to field work.[33]

The suggestion has also been made that titrations of serum may be performed by adding a single dilution of a serum to varying dilutions of virus. The end point would be read in terms of the amount of virus that a specific dilution of serum would inhibit. Since the antibody titer obtained varies inversely with the concentration of hemagglutinin, this method is sound in principle and should give differences between sera of the same order of magnitude as obtained by the technics described above. The method cannot be recommended, however, until it has been more thoroughly investigated and possible pitfalls examined

F. GENERAL CONSIDERATIONS ON THE INTERPRETATION OF SEROLOGIC TESTS

1. *The interpretation of a significant rise in antibody titer.* A significant rise in titer is one which is well beyond the experimental error of the method employed for measurement, and these limits have been already stated. With a significant rise in titer the result is highly diagnostic of infection in the individual tested. So far no false positive results have been reported. A possible exception is the experience of Henle *et al*,[34] who found that human subjects challenged and infected with A virus occasionally developed small rises to influenza B. This may well be true in naturally occurring infections too since it is common to find a small sprinkling of A responses in a predominantly B epidemic or vice versa. One other source of confusion may arise through the use of influenza vaccines in human subjects since the antibody response to parenterally injected virus cannot be differentiated from that due to infection. Where an antibody response to both types of virus occurs, vaccination should be suspected. A person's titer may continue to rise for as long as 3 weeks after vaccination

It must also be recognized that especially during epidemic times, many infections with influenza virus occur which are unaccompanied by clinical symptoms, and these persons will show antibody responses of the same order of magnitude as those who become ill

2. The interpretation of negative serologic results and of responses of less than critical magnitude. Unfortunately, the occurrence of serum pairs which show no change in titer to A or B by no means rules out infection with one of these viruses. During epidemic periods, 60 to 80 per cent of those persons diagnosed clinically as having influenza will show a 4-fold or greater antibody response by any of several methods Many of those who fail to show a response may nevertheless have influenza, and the lack of response may be due to a number of causes:*

a Clinical diagnosis. During nonepidemic periods a large majority of cases which are symptomatically the same as the etiologic entities Influenza A and B, appear to be due to some other, as yet unknown, etiology, and give no response to either A or B No clinical method of separating the etiologically specific cases has been worked out. During epidemic periods, however, the percentage of such nonspecific cases falls far lower, most of the clinical diagnoses are likely to be correct, and failure to corroborate the clinical impression in the laboratory is more likely to be due to one of the following causes

b Poor reactors. During epidemic periods the largest part of the group of cases tested which must be called negative from the laboratory standpoint are those in whom a serologic rise occurs, but the response is less than the critical value necessary for a definite answer The size of this group may be reduced by the use of a method which permits diagnosis on the basis of a 2-fold rise

c Cases with high initial antibody levels This group is similar to the above in showing less than a 4-fold response, but the subjects have a high initial antibody level at the onset of infection, and hence the production of a considerable amount of antibody may be obscured by that already present This is especially true of persons recently vaccinated

d Variation in the specificity of response There is a small group of persons in whom a significant response to infection does not occur when tested with the standard strains but in whom a critical rise may occur when tested with other strains of the virus

e Infection due to other types of influenza virus The postulation of types of influenza virus other than A and B has been made to explain the negative reactors Since 1933 no outbreak of influenza has occurred in the United States in which either influenza A or B did not play a major role It seems unwarranted to postulate further etiologic agents on the basis of the small percentage of individuals who failed to give a specific response, unless all the factors listed above can be ruled out That other etiologic agents may be responsible for many of the endemic cases seems more likely

* If a serum titer drops markedly in 10 to 14 days' time, the probability is excellent that the individual experienced an influenzal infection before the first serum was taken

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G. A RAPID METHOD OF SEROLOGIC DIAGNOSIS FOR EPIDEMIC INFLUENZA

One of the gravest defects in the serologic diagnosis of influenza is the length of time necessary to secure a result. Since the disease is of characteristically brief duration, a diagnosis is not achieved until long after recovery. It is very common, however, for the request for laboratory aid in the diagnosis of influenza to come when an epidemic is in progress, and frequently the request for assistance comes only after the disease has been prevalent for 10 days or more. In this situation it is possible to find a number of individuals who are already convalescent from the disease. A diagnosis of the etiology of the epidemic can frequently be made within a few hours by testing the sera taken from 10 or more convalescent cases and an equal number of those still acutely ill. All of the sera should be titrated in a single test against influenza A and B, and the mean titer for each virus (preferably the geometric mean) calculated for each group. If the disease at hand is influenza, the mean convalescent titer for either type A or B should be significantly higher (4-fold or more) than the acute phase titer mean for the same virus. If neither shows any distinct change in mean level, the disease is probably not influenza.

More information on this type of test is needed, but so far where it has been used the information obtained has been verified by later unequivocal evidence.* If the outbreak occurs in a place remote from a laboratory, field tests may conveniently be carried out on location, and for this the finger-blood technic worked out by Salk [83] is particularly useful.

H. OTHER SEROLOGIC METHODS

1 *Neutralization test in mice* This is the classical method of measuring influenza antibodies and makes use of the fact that the addition of serum antibody to active virus reduces or abolishes its capacity to infect and kill mice (neutralization) The test has been used extensively for diagnostic purposes but will not be described in detail here Serial 4-fold dilutions of heated serum are prepared to which equal volumes of active virus are added The virus suspension has been diluted so that the final mixture contains 100 to 1,000 50 per cent lethal doses of virus per 0.5 ml The virus serum mixtures are inoculated intranasally into Swiss mice under light ether anesthesia, using 0.05 ml per mouse and 4 to 6 mice per dilution The mice begin to die after 4 or 5 days and should

* An excellent example of this is given in a paper by J H Milstone, *et al* [illegible] Geometric mean titers of acute cases were A 194 and B 69, convalescent means were A:169 and B 1100

be autopsied to confirm the presence of pulmonary lesions. The survivors after 7 to 10 days are killed and autopsied for lung lesions. Fifty per cent mortality or lesion end points may be calculated The error of the method when using 4-fold dilutions of serum was estimated as being about 4-fold The technic is slow and costly and does not yield unique information that cannot be found with *in vitro* methods.

2. *In ovo neutralization tests* Antibodies may be titrated *in ovo* using the agglutination test as a rapid check on infection in the individual egg The method has been described fully elsewhere[26, 27] As an *in vivo* method of antibody titration it has the advantage over the mouse test of requiring only 48 hours for completion and permits the use of nonmouse-adapted strains of virus.

IV. SUMMARY

A number of technics applicable to diagnosis of influenza have been described. For laboratories wishing to expand the scope of service offered, influenza diagnosis may make an excellent beginning in the virus field since one may begin with a simple, readily executed serologic technic which does not require special apparatus, and the service may be expanded if desired to include virus isolation procedures, with little added cost and no hazard to laboratory personnel.

V. APPENDIX

A Simplified Method for Determining End Points for the Densitometric Method of Hemagglutinin and Antibody Titration

Most types of photometers give curves with hemagglutinin and antibody titrations which are best suited for interpolation of end points when the serum or virus dilution is plotted on a logarithmic scale. Because of this, some convenient method of rapid conversion of arithmetic to logarithmic dilution values is essential for convenience The usual logarithmic system to the base 10 is fairly cumbersome for this purpose, and the following system has proved to be simple and helpful in several ways.

The method is based on a system of logarithms to the base 2. In any series of 2-fold dilutions (hemagglutinin or serum) the individual tubes are referred to by simple whole numbers The first tube containing undiluted material is tube zero, the 1·2 dilution is tube 1, 1:4 dilution is tube 2, etc. The numbers assigned to the tubes when used as the exponent of 2 become an expression of the arithmetic dilution: for example, the dilution in tube 5 can be expressed arithmetically as 2^5 or 1:32. Intermediate dilutions are expressed as decimals, and thus

a dilution half way between tubes 2 and 3 on a logarithmic scale is 2.5, or $2^{2.5}$, or 5.7. The arithmetic value of such fractional dilutions may be obtained from Table 1.

TABLE 1

Chart for converting log to base 2 figures to arithmetic values
Log_2 dilution = arithmetic dilution

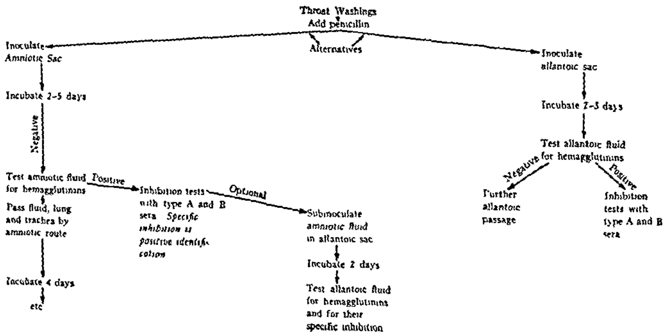
1 0=2.0	2.0=4.0	3 0= 8 0	4.0=16 0	5.0=32
1.1=2.1	2.1=4.3	3.1= 8.6	4.1=17.2	5.1=34
1.2=2.3	2.2=4.6	3.2= 9.2	4.2=18.4	5.2=37
1.3=2.5	2.3=4.9	3.3= 9.8	4.3=19.7	5.3=39
1.4=2.6	2.4=5.3	3.4=10.6	4.4=21.1	5.4=42
1 5=2.7	2.5=5.7	3.5=11.3	4.5=22.6	5.5=45
1.6=3 1	2.6=6.1	3.6=12.1	4.6=24.2	5.6=49
1.7=3.2	2.7=6.5	3.7=13.0	4.7=26.0	5.7=52
1.8=3.5	2.8=7.0	3.8=13.9	4 8=28.0	5.8=56
1.9=3.7	2.9=7.5	3 9=14.9	4.9=30.0	5.9=60
2.0=4 0	3 0=8.0	4 0=16.0	5.0=32.0	6.0=64
6.0= 64	7 0=128	8.0=256	9.0= 512	10 0=1024
6.1= 69	7.1=138	8.1=275	9.1= 549	10.1=1100
6 2= 74	7.2=147	8.2=294	9.2= 588	10 2=1175
6 3= 79	7.3=158	8.3=315	9.3= 632	10.3=1270
6 4= 84	7.4=169	8.4=338	9.4= 676	10 4=1350
6.5= 91	7.5=182	8.5=362	9.5= 722	10.5=1450
6 6= 97	7.6=194	8.6=388	9 6= 779	10.6=1560
6 7=104	7 7=208	8 7=417	9.7= 835	10.7=1670
6 8=112	7.8=223	8.8=447	9.8= 891	10 8=1780
6.9=120	7.9=239	8.9=479	9.9= 958	10.9=1910
7.0=128	8.0=256	9.0=512	10.0=1024	11.0=2048

The method of interpolation may best be given in an example· Suppose that in a hemagglutinin titration, tube 4 reads 146, 5 reads 143, 6 reads 132, 7 reads 78, and 8 reads 56. If the 50 per cent cell concentration reads 100 on this instrument, the end point obviously falls between tubes 6 and 7, and the titer is greater than 1:64 but less than 1:128. For determining the exact end point the proportion is calculated for the difference between the reading on tube 6 and the 50 per cent reading divided by the total difference between the readings of tubes 6 and 7. The former is 132 minus 100 or 32 and the total difference is 132 minus 78 or 54. The 50 per cent point is therefore

32/54ths of the difference between tubes 6 and 7 or as the nearest one place decimal 0.6. The end point in logarithms to the base 2 is therefore 6 0 plus 0.6 or 6 6, which from Table 1 is a dilution of 1:97. The interpolation can be done more quickly with a nomogram.[88] This can be easily constructed in the form of an H on squared paper. Densitometer readings are plotted linearly on the vertical arms of the H, the same way on each side. The crossbar is at the level of the 50 per cent red cell reading and should be divided into 10 equal parts. With a straight edge the reading in the lower dilution is connected on the left with that of the higher dilution on the right, and the nearest figure at which the crossbar is intersected gives the interpolated decimal desired.

The method has other advantages besides facilitating interpolation. One becomes accustomed to thinking and speaking in terms of tube numbers, which are much more convenient than arithmetic dilutions, especially the higher ones. Calculation of the virus dilution necessary for adding to serum dilutions in antibody titrations is simplified. If the titer of a virus suspension is 6 8, the desired dilution is 6.8 minus 2 or 4.8 which from the table is 1:28. A rise of titer of 2-fold is simply a difference of 1 0 on the log to the base 2 scale. If a serum standard is used, its variation from the standard value may be simply added or subtracted from all the logarithmic serum titers obtained, avoiding complicated multiplication or division. The calculation of a geometric mean becomes reduced to the simple operation of adding the $\log_2$ values and dividing by the number of sera tested.

Isolation and Identification of Influenza Virus in Eggs



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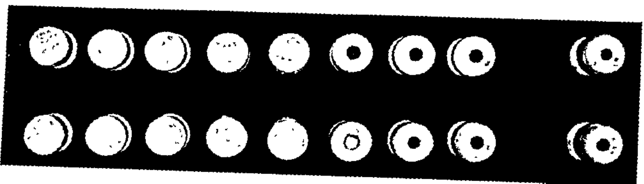


FIGURE 1

FIGURE 1 Photograph of bottom view of tubes, in a titration of agglutinins, showing patterns formed by sedimented cells in two different 2-fold serial dilutions of the same allantoic fluid infected with influenza-virus, type A Each tube contains 0.5 ml of a 0.25 per cent chicken-cell control The characteristic pattern of complete agglutination is seen in the first 5 tubes of each series A partial reaction is illustrated in the 6th tube in the lower row Readings are recorded as follows

	Final Dilutions of Allantoic Fluid								
	160	320	640	1,280	2,560	5,120	10,000	20,000	End point
Top Row	+	+	+	+	+	0	0	0	2,560
	120	240	480	960	1,920	3,840	7,680	15,000	End point
Bottom Row	+	+	+	+	+	±	0	0	1,920

Taken from Salk, J. E.[39]

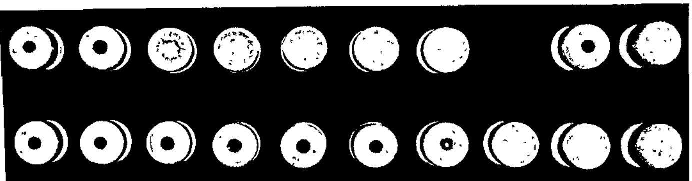


FIGURE 2

FIGURE 2 Photograph of bottom view of tubes in an agglutinin-inhibition titration of an acute and a convalescent serum, showing typical patterns of sedimented cells Serum of the acute phase is in the top row, and the convalescent serum in the bottom row The first tube in each series represents the negative serum-cell control The two tubes in the upper right hand corner of the photograph are the saline-cell control and virus-cell control respectively Readings are recorded as follows

	Control	Final Serum Dilution									Inhibition titer
		32	64	128	256	512	1,024	2,048	4,096	8,000	
Acute	0	0	+	+	+	+	+	+	+	+	32
Conv	0	0	0	0	0	0	±	+	+	+	512

Taken from Salk, J E[38]

PRIMARY ATYPICAL PNEUMONIA

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I. INTRODUCTION

A. Definition

THE SYNDROME, primary atypical pneumonia [1] may be defined as an acute infectious disease of the respiratory tract in which pulmonary infiltration is a prominent feature.[2-6] The pulmonary involvement

is characteristically more evident in roentgenograms than by physical examination. The lesion tends to be patchy and less dense than that seen in pneumococcal pneumonia. Slight or moderate dullness to percussion and the presence of rales constitute the usual physical signs in the chest. The pulse and respiratory rates are either normal or only moderately increased. Constitutional symptoms (headache, malaise, chilliness, and fever) usually predominate over respiratory symptoms early in the illness. Cough is almost invariably present later in the illness. Sputum is mucoid or mucopurulent. The leukocyte count is normal or only moderately increased. As a rule, the illness is of mild or moderate severity and is similar in onset and symptomatology to the moderately severe acute respiratory diseases commonly termed nasopharyngitis, bronchitis, or "grippe." The febrile course is usually remittent, of 3 to 8 days' duration, and terminates by lysis. Occasionally, the disease may be very severe, with extensive pulmonary infiltration, cyanosis, dyspnea, and rapid respiratory and pulse rates. Very mild or even asymptomatic cases have been recognized.

B. ROUTES OF TRANSMISSION

The mode and manner of transmission are not understood. Presumably, the disease is acquired by direct contact or is airborne.[7] Respiratory tract secretions contain the infective agent.[8] It has been suggested that primary atypical pneumonia may represent a severe form of a common, mild epidemic infection of the respiratory tract.[9, 10]

C. DIAGNOSIS

Primary atypical pneumonia can be differentiated from the bacterial pneumonias on clinical grounds in most instances. It is not sound practice, however, to make this differentiation in the absence of adequate bacteriologic studies of the sputum and blood. The absence of such features as shaking chill, pleuritic pain, rusty sputum, frank signs of pulmonary consolidation, and leukocytosis aids in distinguishing the disease from pneumococcal pneumonia. In approximately 10 per cent of the cases the differential diagnosis from pneumococcal or other bacterial pneumonias may be difficult or impossible. This is especially true early in the illness, when a decision is most desirable from the standpoint of therapy. It should be emphasized that this difficulty in differential diagnosis may occur even though proper bacteriologic studies have been made.

The diagnosis of primary atypical pneumonia depends to a large extent upon the exclusion of the pneumonias of known cause. A presumptive diagnosis is usually warranted in the presence of an acute, febrile, respiratory disease with

pulmonary infiltration in which common bacterial agents appear to have been adequately excluded Even under these circumstances, several diseases may present a clinical picture which is exceedingly difficult or impossible to distinguish from primary atypical pneumonia[11, 12] These diseases may be bacterial, such as pulmonary tuberculosis, tularemia, or various bacterial pneumonias; they may be caused by viruses, such as the psittacosis-ornithosis group, influenza A, influenza B, or lymphocytic choriomeningitis; or they may be due to rickettsia, such as Q fever; and, finally, they may be due to fungi, such as coccidioidomycosis and, possibly, histoplasmosis These diseases can ultimately be differentiated from primary atypical pneumonia by employing appropriate laboratory tests* Most of these laboratory procedures either are complicated and require specially trained personnel or are time-consuming because they require acute and convalescent phase sera The diseases just listed, however, are usually responsible for a relatively small proportion of the patients presenting the signs, symptoms, and roentgenographic features of primary atypical pneumonia

Since the agent causing primary atypical pneumonia has not been isolated and characterized, there are no specific laboratory tests available for the diagnosis of the disease Bacteriologic studies have shown that the aerobic bacterial flora of the respiratory tract does not differ significantly from that of other forms of acute respiratory disease or from well individuals[14] The possibility that a bacterium, streptococcus MG may play a role in the causation of the disease[15, 16] has not been entirely excluded. The results of transmission experiments employing human volunteers[6] have shown that the disease can be reproduced by employing bacteria-free filtrates of respiratory tract secretions (sputum and throat washings) Presumably, then, primary atypical pneumonia is caused by a virus. Transmission of a filtrable agent to cotton rats, hamsters, and chick embryos has been reported.[17] Neutralization of this agent by convalescent phase sera from certain patients with primary atypical pneumonia has also been reported[17-19] So far, these technics have not been widely employed, and the data now available do not permit their adequate evaluation.

There are two laboratory tests which are useful adjuncts in differential diagnosis late in the course of illness They are the tests for cold hemagglutinins and agglutinins for streptococcus MG Other serologic reactions have been described in primary atypical pneumonia, but since they are not of value in the diagnosis of the disease, they will be mentioned only briefly

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* See other sections of the present book and Reference 13

II. COLD HEMAGGLUTINATION

A. General Aspects

The basis for this test is the observation that convalescent phase sera from many cases of primary atypical pneumonia contain agglutinins for human group O erythrocytes which are operative at temperatures ranging from 0° to 10° C. With sera of high titer, agglutination may occur at 20° to 25° C. The homologous erythrocytes are also agglutinated in these temperature ranges. At 37° C. the agglutination disappears.

The phenomenon of cold hemagglutination was clearly described by Landsteiner in 1903,[20] employing erythrocytes and sera from normal guinea pigs, chickens, horses, dogs, rabbits, and cattle. He noted the striking effect of low temperature, the disappearance of agglutination with a rise in temperature, the absorbability of the agglutinin in the cold, and its release from the cells at higher temperatures. Clough and Richter in 1918[21] described cold hemagglutinins in high titer in serum from a case of bronchopneumonia. During the last few years, studies of cold hemagglutinins in primary atypical pneumonia have been carried out by Peterson, Ham, and Finland,[22] Turner,[23] and many others.* From these studies several facts have emerged which have an important bearing upon technic and interpretation of the results of tests for cold hemagglutinins in primary atypical pneumonia.

The reported incidence of cold hemagglutinins in cases of primary atypical pneumonia has averaged about 50 per cent,[25] but the figures have varied widely. One factor which may account for this variation is the observation that cold hemagglutination can be correlated with the severity of illness as judged by the symptomatology, the extent of the pulmonary lesion, and the height and duration of the febrile response.[26] [27] The lower reported figures may well have been the result of studies in which a high proportion of the milder illnesses were included. This was apparently true of studies carried out in the military services. In one such study, cold hemagglutinins were found in only about 30 per cent of cases. In civilian hospitals where the more severely ill patients would tend to be preferentially admitted, the occurrence of cold hemagglutinins may be as high as 75 to 90 per cent.

The stage of the illness when the specimens of blood are taken, the temperature at which the blood is kept before the serum is removed, and the length of time and the temperature at which the sera have been stored may affect the results of tests. Cold hemagglutinins are either absent, or are present only in low titer, during the first few days of

* The interested reader is referred to two excellent surveys of the subject, one by Stats and Wasserman[24] and the other by Finland and coworkers[25]

illness, begin to appear either late in the 1st week or during the 2d week, are usually at their maximum in the period from the middle of the 2d week to the middle of the 4th week, and are either absent, or are present in considerably decreased titer from the 4th to 6th weeks. Blood drawn for the purpose of testing for cold hemagglutinins should not be stored in the refrigerator before the serum is separated because the autologous cells will remove the cold hemagglutinins. With serum of high titer, some of the agglutinins may be removed even at room temperature. The cold hemagglutinins, however, will elute from the cells if the blood is thoroughly warmed at 37° C. before the serum is removed. The titer of cold hemagglutinins may decrease in sera stored at 4° C. for long periods; if stored at room temperature, the loss may be quite rapid.[22, 24, 26]

The results of tests for cold hemagglutinins in a given serum may be considerably influenced by the density of the suspension of erythrocytes, by the age of the cells, and by the manner in which the tests are read. When agglutination is read with the unaided eye, a 1 to 2 per cent suspension of cells will usually not show agglutination with "normal" sera in a titer higher than 8 or 16. With a 0.2 per cent suspension of cells the titers of some "normal" sera will range up to 32 or even 64. By using a light suspension of cells and reading the resultant agglutination by means of the microscope, titers in the range of 128 may occasionally be obtained in "normal" subjects Most workers have employed the density which appeared to facilitate the reading of lesser degrees of agglutination There is little agreement concerning the ideal density of erythrocyte suspension to be employed This is unsatisfactory because titers from different laboratories cannot be compared It seems desirable to adopt a density which will permit the highest degree of sensitivity compatible with ease of reading the tests It is suggested that a 0.2 per cent suspension fulfills these criteria[26] Erythrocytes older than 5 or 6 days should not be used since they are less sensitive to agglutination than cells 2 to 4 days old.[illegible] Apparently, fresh cells or cells 1 day old are also somewhat less sensitive than 2- to 4-day cells.[26]

Erythrocytes from certain group O donors are more easily agglutinated than are those from other donors[illegible] There appears to be no way to determine which individuals possess cells that agglutinate readily except by comparative tests. While these differences between donors are detectable, they are not of such a magnitude as to be of major importance With cells 2 to 4 days old, differences between the cells from different donors are minimized.[26]

B Technic of Test

The following technic[26] is recommended.

1. *Sera.* All sera from a patient should be tested on the same day to minimize day-to-day fluctuations in the tests. Sera need not be in-

activated, but inactivation at 56° C. for 30 minutes does not interfere with the test. Sera should be as fresh as possible and must be taken at appropriate times after the onset of illness.

2. *Erythrocytes.* Defibrinated, oxalated, or citrated human group O blood may be employed. The cells are washed 3 times with physiologic saline and then packed in the horizontal centrifuge at 1,500 r.p.m. for 15 minutes. A 0.2 per cent suspension by volume of packed cells in physiologic saline is used in the test. Unused parts of the 0.2 per cent suspension should be discarded each day. The unused part of packed cells may be stored at 4° C in a small volume of physiologic saline.* The suspension of packed cells should be washed again on each day the test is performed.

Two-fold serial dilutions of the sera beginning with a 1 to 4 dilution are made in physiologic saline in 0.5 ml volumes. Tubes measuring 12 by 100 mm are satisfactory. To each tube is added 0.5 ml. of 0.2 per cent suspension of erythrocytes, the final dilution of serum in the first tube is 1 to 8. After thorough shaking, the racks of tubes are kept in the refrigerator at approximately 4° C. overnight. The following morning the tubes are removed from the refrigerator, one rack at a time, and read immediately before warming occurs. A fluorescent source of light and a black background are helpful. Readings are graded from 4+, which consists of a tight disc of cells which does not break up readily on gentle shaking of the tube, to 1+, which is the least amount of definite clumping visible to the unaided eye. The end point is the highest final dilution of serum in which definite (1+) agglutination occurs. Positive tests must be read for the disappearance of agglutination after 30 minutes at 37° C. to eliminate hetero-agglutinins or other antibodies which might cause agglutination. Titers of cold hemagglutinins are expressed as the reciprocal of the final dilution of serum at the end point. If available, positive sera of previously determined titer should be included in each day's tests as a check on the sensitivity and reliability of the tests from day to day. Titers of the control sera should not vary more than ± 1 dilution from day to day.

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* Finland, Samper, and Barnes[44] have reported that cells stored in the original plasma gave higher titers and clearer end points than cells which were stored as a 2 per cent suspension in physiologic saline.

C. INTERPRETATION OF RESULTS

Employing the above technic, titers of 32 or 64 may be considered abnormal. High titers—128 to 1,024 or more—are not often seen except in primary atypical pneumonia or in certain hemolytic anemias. More important than the titer of a single serum is the demonstration that an increase in titer has occurred during the course of illness. A 4-fold or greater increase in titer is probably significant. Likewise, a definite decrease in titer late in convalescence is important. To detect such changes in titer, it is important that the sera be collected at appropriate intervals (see above).

Cold hemagglutinins have been described in trypanosomiasis, blackwater fever, mumps orchitis, hemolytic anemias, certain diseases of the liver, peripheral vascular disease, and so on.[26] Most of these diseases are not too difficult to differentiate from primary atypical pneumonia. *Cold hemagglutinins, usually in low titer, may also occur in a variety of the common respiratory diseases.*[24, 31-34] However, experience has shown that primary atypical pneumonia is the *only* respiratory disease which is likely to lead either to a large increase in titer in serial sera or to the presence of a high titer of cold hemagglutinins in a single serum specimen taken at the proper time after the onset of illness.

It should be emphasized that the detection of cold hemagglutinins is not pathognomonic of primary atypical pneumonia and that failure to demonstrate the presence of the agglutinins does not exclude the diagnosis. Since these agglutinins do not appear until late in the course of illness, the value of the test in differential diagnosis is usually retrospective.

III. AGGLUTININS FOR STREPTOCOCCUS MG*

A. GENERAL ASPECTS

Streptococcus MG[36] is a nonhemolytic streptococcus which appears to be distinct serologically from other species of streptococci. The organism possesses a capsular structure which is responsible for its type-specific immunologic reactions. It is immunologically related to, but antigenically distinct from, *Streptococcus salivarius*, type I.

Streptococcus MG is not easily distinguished from other varieties of indifferent streptococci which inhabit the respiratory tract, notably *Streptococcus salivarius*, type I, and *Streptococcus mitis*. However, it has been found[36] that

* Formerly called streptococcus 344

activated, but inactivation at 56° C. for 30 minutes does not interfere with the test Sera should be as fresh as possible and must be taken at appropriate times after the onset of illness

2. *Erythrocytes.* Defibrinated, oxalated, or citrated human group O blood may be employed. The cells are washed 3 times with physiologic saline and then packed in the horizontal centrifuge at 1,500 r p m. for 15 minutes A 0 2 per cent suspension by volume of packed cells in physiologic saline is used in the test. Unused parts of the 0.2 per cent suspension should be discarded each day. The unused part of packed cells may be stored at 4° C. in a small volume of physiologic saline.* The suspension of packed cells should be washed again on each day the test is performed.

Two-fold serial dilutions of the sera beginning with a 1 to 4 dilution are made in physiologic saline in 0.5 ml volumes Tubes measuring 12 by 100 mm. are satisfactory. To each tube is added 0 5 ml. of 0.2 per cent suspension of erythrocytes; the final dilution of serum in the first tube is 1 to 8 After thorough shaking, the racks of tubes are kept in the refrigerator at approximately 4° C overnight. The following morning the tubes are removed from the refrigerator, one rack at a time, and read immediately before warming occurs A fluorescent source of light and a black background are helpful. Readings are graded from 4+, which consists of a tight disc of cells which does not break up readily on gentle shaking of the tube, to 1+, which is the least amount of definite clumping visible to the unaided eye The end point is the highest final dilution of serum in which definite (1+) agglutination occurs. Positive tests must be read for the disappearance of agglutination after 30 minutes at 37° C. to eliminate hetero-agglutinins or other antibodies which might cause agglutination. Titers of cold hemagglutinins are expressed as the reciprocal of the final dilution of serum at the end point. If available, positive sera of previously determined titer should be included in each day's tests as a check on the sensitivity and reliability of the tests from day to day Titers of the control sera should not vary more than ± 1 dilution from day to day.

* Finland, Samper, and Barnes[30] have reported that cells stored in the original plasma gave higher titers and clearer end points than cells which were stored as a 2 per cent suspension in physiologic saline.

the severity of illness. In mild cases of primary atypical pneumonia the agglutinins may be present in only about 20 per cent; in the more severe or prolonged illnesses they may be found in approximately 75 per cent of cases. Immunologic studies have indicated that cold hemagglutinins and agglutinins for streptococcus MG are not related.[37] The observation that a positive test for streptococcus MG agglutinins is more likely to be found in a patient who has developed cold hemagglutinins may be explicable, at least in part, by the fact that each occurs more abundantly in severe cases.

B. Technic of Test

1. *Isolation of streptococcus MG.* Isolation of streptococcus MG may be satisfactorily accomplished by employing the technic devised by Thomas, Mirick, Curnen, Ziegler, and Horsfall.[37] Their technic follows:

A loopful of sputum is inoculated into approximately 4 ml of semiselective medium and is incubated at 37° C for 24 to 48 hours. Throat swabs may be placed directly in 4 ml of the medium and incubated. The medium is composed of the following constituents:

Proteose peptone, Difco	5 gms
Yeast extract, Bacto	5 gms
Beef extract, Bacto	3 gms.
Glucose C P	10 gms
Gentian violet	2 mgm
Sodium azide	200 mgm
Sulfapyridine	500 mgm
Distilled water	1000 ml

The pH is adjusted to 7.2 with 1 N NaOH

Sterilize in autoclave at 15 pounds pressure for 15 minutes

Lung tissue suspension should be cultured in Brewer's thioglycollate medium.[43] When visible growth has occurred, a loopful of culture is mixed with a loopful of rabbit antistreptococcus MG serum, together with a loopful of 1 per cent methylene blue. A positive Quellung reaction identifies an organism as being either streptococcus MG or *Streptococcus salivarius*, type I. To differentiate between these organisms, the culture is inoculated on the surface of 5 per cent sucrose agar plates. *Streptococcus salivarius*, type I, forms large succulent

streptococcus MG produces small fluorescent colonies on sucrose agar whereas *Streptococcus salivarius*, type I, produces large succulent colonies Various strains of *Streptococcus mitis* will ferment raffinose and produce marked greening on blood agar. These properties are not possessed by streptococcus MG, although definite but small degrees of green hemolysis may occur.

Streptococcus MG was first isolated from the lungs of fatal cases of primary atypical pneumonia.[36] It has since been isolated from the upper respiratory tracts of normal persons and of persons with non-pneumonic acute respiratory disease.[34, 37] The organism, however, has been isolated from the respiratory tract secretions and lungs of cases of primary atypical pneumonia more frequently than from the other forms of acute respiratory disease.[15] The part which streptococcus MG plays in the mechanism of primary atypical pneumonia is unknown.* In the experiments in which primary atypical pneumonia was transmitted to human volunteers, streptococcus MG was isolated with almost identical frequency in the total group of volunteers before as well as after inoculation.[34]

More pertinent to the present discussion is the observation that many patients convalescent from primary atypical pneumonia develop antibodies in their blood for streptococcus MG. These antibodies may be demonstrated by agglutination of either the encapsulated organisms or nonencapsulated R variants, by precipitation with the capsular substance, by capsular swelling, or by skin reactions on intradermal injection of the capsular substance.[37] Only the agglutinins will be considered here because the technic for their detection appears to be the most practical for use in most laboratories and is the only one which has received sufficient usage to permit of its evaluation.[37–42] Although these agglutinins do not appear in all cases of primary atypical pneumonia and may be found in certain other conditions, their detection in convalescent phase sera is of value in the differential diagnosis of primary atypical pneumonia. Since the antibodies do not appear until the 2d or 3d week after the onset of illness and reach maximum levels during the 4th and 5th weeks, the demonstration either that they are present in high titer or that a significant increase in titer has occurred is of value only in making a retrospective diagnosis As in the case of cold hemagglutinins, antibodies for streptococcus MG can be correlated with

* The interested reader is referred to two recent papers which discuss the role of streptococcus MG in primary atypical pneumonia.[33, 35]

streptococcus MG may occasionally occur at icebox temperatures. Such agglutination disappears when the mixtures are brought to 37° C.

In estimating the degree of agglutination, the following standards are employed A designation of 4+ is assigned to tubes in which agglutination is complete, with a solid plaque or disc of bacteria and a clear supernatant fluid Agglutination with large clumps and clear supernatant fluid, but without complete settling of the bacteria to the base of the tube, is designated as 3+. Agglutination with incomplete clearing of the supernatant fluid is designated as 2+. Agglutination with turbid fluid, but with particles visible to the unaided eye, is designated as 1+. Agglutination which requires the use of a hand lens for visualization is designated as ±. The agglutination titer is taken as the highest dilution of serum in which reactions of 1+ or more are observed.

C Interpretation of Results

Titers of 1:20 or more may be found in approximately 50 per cent of cases of primary atypical pneumonia[15] However, the reported incidence has varied widely,[18] and in one series of cases was less than 10 per cent[43] As noted above, the agglutinins appear to occur more commonly in the more severe cases The agglutinins may be found in normal persons, in various acute respiratory infections, and in certain streptococcal infections[37-41] They are usually present in low titer in these conditions[15]

As in the case of cold hemagglutinins, the demonstration of an increase in titer of a 4-fold increment or more during convalescence is more important than the height of the titer in a single serum specimen. Recently, it has been pointed out that a significant increase in titer of streptococcus MG agglutinins has not been demonstrated in any condition other than primary atypical pneumonia[45]

Several features concerning the interpretation of the results of tests for agglutinins to streptococcus MG must be kept in mind

1 Sera must be obtained at the proper stage or stages of illness

2 Low titers (1 10 or 1 20) must be interpreted with caution.

3 While significant increases in titer have been found predominantly, or solely, in primary atypical pneumonia, experience with the test has been limited

colonies whereas streptococcus MG forms small fluorescent colonies The latter colonies are inoculated in beef infusion broth, and the resultant growth tested for the Quellung reaction in rabbit antistreptococcus MG serum. For continued subculture of streptococcus MG in the laboratory, Todd-Hewitt broth[46] is employed When cultures are stored at 4° C for more than a week, defibrinated rabbit blood should be added to the broth in a concentration of 2 per cent. Blood broth cultures remain viable for as long as 2 months at 4° C

When rabbit antistreptococcus MG serum is not available, the cultures in the semiselective medium may be plated directly on the 5 per cent sucrose agar plates The small fluorescent colonies may then be subcultured and suspensions prepared for agglutination tests with sera from cases of primary atypical pneumonia. By this method streptococcus MG can be identified with a reasonable degree of assurance when an organism is found which is agglutinated by convalescent phase sera to a titer at least 4 times greater than acute phase sera

2 *Materials and methods for agglutination test.* The technic[47] is as follows:

Bacterial suspensions for agglutination tests are prepared from 18-hour broth cultures of streptococcus MG The bacterial cells are washed 3 times in 0 85 per cent NaCl and suspended in sufficient physiologic saline to give a turbidity approximating No 5 in the McFarland scale. The streptococci are killed by heating at 65° C for 1 hour Merthiolate, in a final concentration of 1 10,000, is added as a preservative.

Serial 2-fold dilutions of unheated serum are made in physiologic saline in 0 5 ml volumes Each serum dilution is then mixed with an equal volume of streptococcal suspension The final dilutions of serum should range from 1 10 to 1 320 Dilutions lower than 1 10 are not used in routine tests because of the frequent occurrence of nonspecific agglutination of streptococcus MG in 1.2 or 1 4 dilutions of either normal human or rabbit serum Sera are not heated at 56° C, since the agglutination titer of convalescent serum may be reduced by heating at this temperature The tubes containing the serum and the streptococcal suspension are placed in a water bath at 37° C for 2 hours, followed by 18 hours in the icebox at 4° C They are then again placed in the water bath at 37° C for 2 hours, after which the tubes are shaken and readings of the degree of agglutination are made The final period of 2 hours at 37° C is of importance since nonspecific agglutination of

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4. The absence of the agglutinins does *not* exclude primary atypical pneumonia.

5. Since the agglutinins do not usually appear until late in convalescence, the results of the tests are of value principally in the retrospective differential diagnosis of primary atypical pneumonia.

IV. OTHER SEROLOGIC REACTIONS

The capacity of convalescent phase sera of cases of primary atypical pneumonia to fix complement with a variety of antigens, particularly those consisting of fresh tissue suspensions, has been demonstrated.[40] The substances responsible for these reactions appear to be separable from those causing cold hemagglutination and agglutination of streptococcus MG.[37] False positive serologic tests for syphilis have also been noted in primary atypical pneumonia.[42] Such serologic reactions have not been demonstrated to be of practical value in the diagnosis of primary atypical pneumonia

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MUMPS

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I. CLINICAL FEATURES OF MUMPS

FOR REVIEWS of the clinical and epidemiologic aspects of mumps, see References 1, 2, 3, and 4a.

A Mode of Transmission

Mumps is transmitted from one individual to another by saliva containing the virus. Transmission may be through direct contact, air-suspended droplets, or fomites recently contaminated with saliva

B. Pathogenesis

It is not known whether the virus at first reaches the salivary glands directly via the duct or whether it enters by another route, such as the tonsil or the olfactory nerve endings, giving rise primarily to either a generalized or an inapparent infection of the central nervous system, with subsequent localization in the salivary glands and other organs

C Incubation Period

In most instances 18 to 21 days elapse between the time of exposure and the first detectable enlargement of the salivary glands.

D. Period of Infectivity

The period of infectivity has not been exactly defined In recent experiments virus has been demonstrated in the saliva on the 5th day following the onset.[4b] The period of infectivity is unkown,[5, 6, 7] if it exists, in cases of inapparent infection, or in cases of meningo-encephalitis, orchitis, and other conditions in which no salivary gland involvement can be discerned

E Symptoms of Uncomplicated Mumps

Uncomplicated infection of the salivary glands is manifested by enlargement of one or more of these organs—usually the parotids The swelling in most cases reaches a maximum after 48 hours, and a gland usually remains enlarged from 7 to 10 days

Fever of short duration and of moderate degree (100° to 101° F) may be present, sometimes the rise in temperature may be negligible or absent.

- B In the Embryonated Hen's Egg
  - 1. Procedures for serial passages
    - a Amniotic sac inoculation
    - b Yolk sac inoculation
    - c Allantoic sac inoculation
  - 2 Procedures for primary egg passages
    - a Inoculation of infected monkey parotid gland
    - b. Inoculation of saliva
  - 3 Mode of harvesting materials containing the virus
  - 4 Indications that infection has occurred
    - a Death of embryo
    - b. Pathologic changes
    - c Presence of hemagglutinin
    - d Presence of complement-fixing antigen

VI CERTAIN PROPERTIES OF THE EGG-ADAPTED VIRUS
- A Size as Measured by Filtration
- B Thermoresistance
- C Persistence on Storage
- D Effect of Certain Chemical and Physical Agents

VII DIAGNOSTIC PROCEDURES DEPENDING UPON THE DEMONSTRATION OF SPECIFIC ANTIBODY
- A Complement Fixation Test
  - 1 Applications
  - 2 Titration of antigens
  - 3 Blood sera and other fluids
  - 4 Complement
  - 5 Hemolytic system
  - 6 Procedure employed in the complement fixation test
  - 7 Interpretation
    - a Complement-fixing antibody as an index of immunity
    - b Complement fixation test as a diagnostic procedure
- B Inhibition of Hemagglutination
  - 1. Applications
  - 2 Technic
    - a Titration of hemagglutinin
    - b Titration of antihemagglutinin
  - 3 Interpretation
  - 4. Comparison of results obtained by complement fixation test and test for antihemagglutinin
- C Virus Neutralization Test in the Embryonated Egg

VIII. INTRACUTANEOUS TEST FOR THE DETERMINATION OF RESISTANCE OR SUSCEPTIBILITY
- A Preparation of the Material
- B Technic of Inoculation
- C Character of the Reaction
- D Interpretation

IX. REFERENCES

in diagnosis even were it practicable to obtain tissues for routine examination.[46]

III. DEVELOPMENT OF LABORATORY DIAGNOSTIC TECHNICS

Until the last few years, no specific laboratory methods have been available for the diagnosis of mumps or its complications. As a result of the investigations of workers in this country and in Australia, however, technics have been provided whereby not only infection by the virus may be diagnosed in the laboratory, but the status of the individual in respect to susceptibility or immunity may be evaluated with a reasonable degree of accuracy.

The nature of the etiologic agent was uncertain until 1934 when Johnson and Goodpasture[21] conclusively demonstrated its viral nature by producing typical parotitis in rhesus monkeys after intraparotid inoculation of saliva from patients with mumps In 1942, Enders and Cohen[29] showed that the infected gland of the rhesus monkey could be successfully employed in a complement fixation test for the demonstration of a specific antibody which developed regularly in the sera of man and monkey convalescent from mumps as well as in certain human beings who had experienced the disease from *5 to 30* years previously Enders and his coworkers[*, 30] also found that when the infectivity of the virus in such antigen was removed by heating the material when inoculated intracutaneously induced a delayed type of hypersensitive response in individuals who had undergone an attack of mumps In 1945 Habel[20] and then Levens and Enders[23] succeeded in cultivating the monkey-adapted mumps virus in the developing hen's egg At *the same time, Levens and Enders[23] found an additional means for detecting the* presence of specific antibody or the virus by showing that the virus possessed a hemagglutinative factor analagous to that of the influenza and other viruses. A third method of determining and measuring antibody appears to have become available in the recent discovery by Burnet[19] that human red cells treated with infected embryonic fluid from which the virus is then removed by elution are specifically agglutinated by mumps convalescent sera In 1946, Beveridge, Lind, and Anderson[20] successfully cultivated the virus in the embryonated hen's egg after the direct inoculation of saliva from patients with mumps

IV. COLLECTION OF MATERIALS EMPLOYED IN LABORATORY DIAGNOSIS

No special precautions need usually be taken in the handling of infected materials If the worker, however, is known to be susceptible†

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* Descriptions of such changes are given in the following papers

Monkey—Parotid Johnson and Goodpasture[21] and Bloch.[12] Testis, Findlay and Clarke[38] Central nervous system, Johnson and Goodpasture[21]

Man—Parotid, Dopter and Repaci[16] Testis, Smith[14] and Manca[14] Pancreas, van Rooyen and Rhodes.[46] Central nervous system, McKaig and Woltman[49] *and* Wesselhoeft[3]

† For methods of determining susceptibility, see Sections VII, A, and VIII

F. COMPLICATIONS

The two most common complications, orchitis (about 18 per cent) and meningoencephalitis (0 5–10 per cent) often develop between the 2d and 10th day after the onset of parotitis But these conditions may also appear either before or at the same time the salivary glands become enlarged. Moreover, these manifestations may at times be the only presenting signs.

Ovaritis has been recorded as occurring in about 5 per cent of adult females with mumps. Other complications, such as pancreatitis and neuritis of various nerves (facial, trigeminal, optic, and auditory) as well as infections of the eye (conjunctivitis, keratitis, iritis, retinitis), or of the inner ear are encountered more rarely.

G WHITE CELL COUNT

1. *In blood* In many cases of mumps uncomplicated by secondary bacterial infection, the total white cell count may be moderately elevated, but in others it is within normal limits, and in some may be depressed. Differentially, there is frequently, but not invariably, an absolute or relative increase in lymphocytes which may be encountered from the 1st to the 14th day of the disease. It is thus evident that the white cell count is of little importance in the diagnosis of infection due to this virus.

2 *In spinal fluid* In mumps meningoencephalitis, the white cell count of the spinal fluid may range from 8 to 10 cells to more than 2,000, 80 to 100 per cent of which are lymphocytes.[6] The mean total count in 11 cases recently studied [9] was 434

In this connection, it should be recalled that in clinically uncomplicated parotitis as well, an increase in the white cells in the spinal fluid has often been observed Total counts entirely comparable to those encountered in frank meningoencephalitis have been recorded.

Aside from the cell content, the spinal fluid in mumps encephalitis shows little significant variation from the normal The proteins and sugar are somewhat increased, the chlorides may be slightly decreased. According to Wesselhoeft,[2] the colloidal gold curve is either normal or conforms to a meningitic reaction

II PATHOLOGY

The pathologic changes in the salivary glands or in other organs which may be affected are not sufficiently characteristic to be of value

of blood at an appropriate interval *in* order to demonstrate the development or increase of specific antibody. Accordingly, blood specimen should be obtained as early as possible after the onset of symptoms, and, if only one other specimen is to be tested, again after an interval of about 14 days. Usually at this time antibody concentration is high. It is preferable, however, to obtain an additional blood sample about 7 days after the onset, when many individuals will have developed an appreciable titer. Thus an earlier diagnosis may be made. If no antibody is demonstrated in the specimen taken on the 14th day, one should be secured on the 21st day, by which time the antibody has been found in all cases so far studied.

V. PROPAGATION OF VIRUS

A. IN THE MONKEY

1 *Technic of inoculation* The procedure of inoculating the monkey via Stensen's duct is described in detail by Johnson and Goodpasture[11] By means of a 24-gauge hypodermic needle from which the point has been removed, the orifice of Stensen's duct is entered This lies adjacent to the papilla situated on the buccal mucous membrane, at a point approximately opposite the 1st upper molar About 2 ml of the crude diluted saliva is gently forced into the gland Visible swelling of the gland or its increase in size as revealed on palpation during the process of inoculation will indicate whether or not the material has been successfully introduced The bacteria that contaminate saliva rarely persist in the gland or cause an infection

2 *Criteria of infection* Although some animals may respond to infection by a rise of temperature and leukopenia, these manifestations have not been constant features of the experimental disease Dependence is placed upon the fulfillment of two or more of the following criteria of infection In most instances, those mentioned under *a*, *b*, and *c* have been regarded as sufficient evidence for the nature of the disease

a Swelling of parotid gland Enlargement of the inoculated parotid gland due to the infection is not observed in normal monkeys until the 6th or 7th day following the introduction of virus Thereafter, in typical cases, increase in size of the gland occurs rapidly, accompanied by marked edema of the surrounding tissues These manifestations, in most instances, reach their height on the 8th or 9th day and then rapidly subside The degree of enlargement of the gland after inoculation of saliva may be minimal and may be accompanied by little or no edema On subsequent passages, however, definite swelling of varying degrees is a constant feature

b Demonstration of complement-fixing antigen in the parotid gland Complement-fixing antigen is nearly always present and in maximal concentration in the parotid gland on the 5th to the 6th day after inoculation Its demonstration, therefore, is a reliable criterion of infection

and is concerned about getting the disease, then precautions should be taken to avoid the introduction of infected material into the mouth or respiratory tract, since a laboratory infection has occurred following the inadvertent contamination of the mouth with infected monkey gland.[8]

A. FOR ISOLATION OF VIRUS FROM SALIVA

With rare exceptions,* the virus of mumps has been demonstrated only in the saliva from patients with parotitis In most instances, then, if a diagnosis is to be made through the isolation of the virus, usually for experimental purposes, specimens of saliva should be collected as early as possible after the onset. It is not yet known whether the virus may temporarily disappear following the subsidence of the initial parotitis of one gland to reappear in the saliva after an interval of a few days when the other gland may become enlarged.

Saliva is collected over a period of 1 to 2 hours An equal quantity of infusion broth is then added, and the material either prepared at once for inoculation as described below or, if necessary, kept at about 4° C for not more than 2 to 4 hours If prolonged storage is necessary the material should be placed in pyrex glass containers, and a glass seal established. After freezing rapidly in a dry ice-alcohol mixture, it should then be stored in a CO_2 ice cabinet

B. FOR SEROLOGIC TESTS

Although attempts to isolate mumps virus are warranted in special circumstances, the laboratory diagnosis of the disease is most readily accomplished, in general, by serologic methods

Approximately 5 ml. of blood are obtained. The blood is taken under sterile conditions and allowed to clot firmly. The serum should then be removed as soon as possible. Precautions to avoid hemolysis should be carefully observed. The serum should be placed in closed containers. It may be stored at 4° C. for not more than 4 weeks. After this period the antibody concentration in human serum may decline significantly.† For this reason, known positive and negative human sera to be used as controls in the complement fixation tests should be preserved in the CO_2 cabinet in glass-sealed vials. Under these conditions the concentration of antibody remains constant. If a specimen is to be shipped, it should be in the form of serum Spinal fluid as well as other body fluids, such as hydrocele fluid, should be kept free of bacterial contamination and treated in the same manner as blood serum

It is essential for a final diagnosis to obtain at least 2 specimens

* For example, Swan and Mawson[34] in 1943 reported the isolation of mumps virus in the monkey from the spinal fluid of 1 out of 4 patients with mumps meningo encephalitis Henle and McDougall[30] recently reported isolation of virus from spinal fluid in two cases by inoculation of chick embryos

† The antibody in monkey serum appears to be more stable[22]

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* [illegible] reported the isolation of mumps virus [illegible] f 4 patients with mumps meningo- [illegible] orted isolation of virus from spinal [illegible]

† [illegible] be more stable[26]

sac. Inoculation of the chorioallantoic membrane employing the technic of Burnet[27] has proved successful but does not appear to offer any advantage. Following intravenous injection, probably little multiplication of the virus occurs since Beveridge and his associates[25] found no hemagglutinin in the amniotic fluid under these circumstances. Embryos may be used which have been developed for 6 to 8 days at 39° C. The atmosphere of the incubator is humidified during this interval as well as during the period subsequent to inoculation, when the temperature of incubation is dropped to 35° C. The optimal postinoculation period of incubation, irrespective of the route of inoculation, is 5 to 7 days. Varying yields of complement-fixing antigen and hemagglutinin may be obtained from the 3d to the 12th day after inoculation

a. Amniotic sac inoculation. The egg is candled, the position of the embryonic eye located, and a slit about ⅛ inch long is drilled approximately ¾ inch above the eye over the air space. After wiping the slit with tincture of iodine and 70 per cent alcohol, the egg is again placed over the candling light in an almost horizontal position. The volume of inoculum is 0.1 ml. By means of a sharp 1½ inch, 25 gauge hypodermic needle, the inoculation is made through the slit with a swift thrust toward the embryo. A lively movement of the latter indicates that the amniotic sac has been penetrated After removal of the needle, the area of injection is again disinfected, and the opening sealed with fingernail polish.

b. Yolk sac inoculation The egg is candled to locate the boundary of the air space From the shell over the air space a triangular section with sides about 1 cm. long is removed. A drop of sterile mineral oil may be placed on the shell membrane at the bottom of the air space to render it more transparent The inoculum in a volume of 0 5 to 1.0 ml. is then introduced into the yolk sac by means of a 1½ inch, 22-gauge needle Because of the large volume, the injection is made slowly to avoid trauma The triangular opening is then sealed with scotch tape sterilized in the autoclave. The egg is incubated in a vertical position with the seal uppermost

c Allantoic sac inoculation After determining and marking the position of the embryonic eye, a slit ⅛ inch long is drilled a little to one side of the mark. Through this aperture, the sac is entered, employing a ½ inch 26-gauge needle, which is inserted at a sharp angle to the plane of the shell. The position of the needle when the injection is made is almost parallel to the shell The volume of inoculum is 0.1 ml.

Infected gland has proved unsatisfactory as a source of hemagglutinin since it has not exhibited the capacity to agglutinate most lots of chicken erythrocytes which are, however, agglutinated by infected amniotic fluid (*see* Section V, B, 4, c).

c. Confirmatory serologic tests

(1). Demonstration of complement-fixing antibody. Complement-fixing antibody appears in all animals inoculated with active virus and tested between the 10th and 14th day. For its demonstration, a blood sample should be taken at the time of inoculation and at least once again on about the 14th day, when titers corresponding to those found in convalescent human serum should be encountered if the inoculum contained virus (*see* Section VII, A, 7, b).

Specimens of serum are obtained before inoculation because monkeys in contact with others in the acute stage of mumps may undergo an inapparent disease that results in the development of complement-fixing antibody.[23]

(2). Demonstration of antihemagglutinin. A serum factor which inhibits the agglutination of red cells by the virus likewise develops regularly following infection[20] A sample of serum should be obtained before inoculation and again on about the 18th day thereafter, and tests for the inhibiting antibody should be carried out according to the technic described in Section VII, B, 2. The 18th day is recommended for obtaining the 2d specimen of serum because the antihemagglutinin may at times appear after the complement-fixing antibody

d. Tests for active immunity Johnson and Goodpasture have shown that monkeys which had recovered from mumps failed to respond with typical symptoms when reinoculated with the virus via Stensen's duct.[11] The absence of typical symptoms, then, following the introduction of 2 ml of a 5 per cent suspension of parotid gland shown, by inoculation into a normal monkey, to contain active virus is evidence of active immunity resulting from the primary inoculation.

The development of a firm, hard swelling of the gland within 24 to 72 hours after the inoculum is administered is further evidence of the immune state. This accelerated response which follows in a high proportion of resistant monkeys is in all probability a manifestation of hypersensitivity to the virus The accelerated reaction may persist for several days but is rarely discernible after the 6th day following inoculation.

e Serial passage of virus. The maintenance through several salivary gland passages of the virus is essential for its complete identification The gland is removed at the optimal time as defined above and tested for the presence of complement-fixing antigen The procedure should be repeated until typical swelling and edema are regularly produced.

B. IN THE EMBRYONATED HEN'S EGG

The following procedures have been successfully employed in our laboratory and are essentially similar to those used by other workers.[20, 23]

1. *Procedures for serial passages.* Three routes of inoculation have proved most useful: the amniotic sac, the allantoic sac, and the yolk

The egg is then placed in a horizontal position with the embryo uppermost, and the overlying shell removed. The amniotic fluid is drawn off by catching a fold of the amniotic sac with a pair of round-end forceps and by entering it with a capillary pipette The yield of fluid from infected embryos which have been incubated for a total period of 13 to 14 days varies considerably. On the average the volume obtained is from 0 25 to 0.5 ml Larger yields are derived from eggs which have been incubated 11 to 12 days. If it is desired to avoid contamination with albumin, the amniotic fluid should be harvested before the 12th day, since at this time the albumin begins to enter the amniotic sac [28]

The remaining contents of the egg, with the exception of the chorioallantoic membrane which is retained within the eggshell by pressure with the forceps, are deposited in a sterile petri dish, taking care to separate forcibly the chorioallantoic membrane from the amniotic in the area of the point of attachment, since once the membranes are intermingled in the petri dish, it is impossible to distinguish them. The amniotic membrane is then stripped from the embryo If, during the above procedures, the embryo should have slipped out of its enveloping membrane, the latter can be located by following along the tissue from the point of attachment to the embryo Frequently the amniotic membrane covers part of the surface of the yolk sac and must be carefully teased away Because the weight of individual membranes is small, varying from 0 2 to 0 5 gm , a pool is made of 4 or 5 membranes. The tissues are washed in about 10 ml of physiologic salt solution and their wet weight determined The yolk sac can be ruptured with the forceps in order to release the larger part of the yolk Much of the remaining yolk can be removed by washing the sac 3 or 4 times in sterile saline The wet weight of individual yolk sacs varies from 1.5 to 3 5 gms Of both the amniotic and the yolk sacs, a 20 per cent suspension is made by grinding the material in a mortar with sterile alundum and saline After centrifugation at about 1,500 r p m for 10 minutes, the supernatant is preserved for further testing Suspensions of chorioallantoic membrane and of the embryo may be prepared in a similar manner Tests for bacterial contamination are immediately carried out on each pool of material All materials can be stored in the icebox overnight until the cultures for sterility are examined. If storage is to be maintained for a prolonged period, it is best to keep the material in glass-sealed pyrex vials in the CO_2 cabinet.

2. Procedures for primary egg passages.

a. *Inoculation of infected monkey parotid gland.* *Virus in the form of infected* monkey parotid may be adapted with little difficulty to cultivation in the egg. Inoculation of a 5 or 10 per cent suspension of infected gland in volumes ranging from 0.1 to 1.0 ml. into the yolk sac has proved successful in the hands of three groups of workers[30, 31, 33] Materials may be harvested from the 5th to the 9th or 10th day thereafter, but the optimal period of incubation can be taken as 6 or 7 days. The test for complement-fixing antigen in the yolk sac may not be positive with material obtained from the 1st passage, but under these circumstances *the antigen is usually found in a suspension of amniotic sac.* Hemagglutinin is usually present in the amniotic fluid of the 1st passage, but in our experience is absent in the allantoic fluid of the same embryo.

b. Inoculation of saliva. Successful isolation of mumps virus directly from the saliva of patients has been reported by Beveridge, Lind, and Anderson[33] and Leymaster and Ward[45] The former were able to cultivate the virus by means of yolk sac inoculation in 3 out of 7 cases. Later the virus was isolated from another patient by inoculating the saliva into the amniotic cavity. The procedures were as follows:

Saliva was diluted with about ⅓ its volume of infusion broth and centrifuged for 5 minutes at 2,500 r.p.m. After removal of the supernatant fluid, centrifugation was repeated. Sufficient penicillin was added to the supernatant to give 300 units per 0.5 ml. The mixture in a volume of 0.5 ml. was inoculated into the yolk sac of 5-or 6-day embryos. Either just before or after the inoculation, 0.4 ml. of 5 per cent sodium sulfadiazine or sodium sulfamerazine was injected into the yolk sac. The volume of inoculum which was injected into the amniotic cavity was not defined, but the standard quantity of 0.1 ml. might well be employed.

Material for further passage was harvested from the 3 or 4 embryos which, of the 6 or 8 incoluated, survived the longest. Employing the yolk sac as antigen, positive complement fixation tests were not obtained in any instance until the 2d or 3d passages in embryos inoculated into the yolk sac. In the successful isolation following inoculation of the amniotic cavity, hemagglutinin was demonstrated in the amniotic fluid of the primary passage on the 4th day.

3. Mode of harvesting materials containing the virus. The egg is candled, the base of the air space marked, and the position of the embryo redetermined if possible. Approximately ⅓ to ½ of the shell overlying the air space is removed. The shell membrane at the base of the air space is carefully stripped off. With the egg in an upright position and with the use of a capillary pipette, the allantoic fluid is drawn off, taking care to avoid injury to large blood vessels with consequent contamination *of the fluid with erythrocytes. If contamination* by red cells does occur to a moderate or marked degree, the fluid is not added to the pool derived from other embryos.

proved more regularly hemagglutinative. At present, however, the routine use of amniotic membrane suspensions as hemagglutinin is not recommended.

d. Presence of complement-fixing antigen. The method of complement fixation for determining the presence of antigen was the first to be employed as an index of infection in the embryo It is, as noted above, a somewhat more reliable procedure than the test for the presence of hemagglutinin.

In the authors' experience the virus appears to have a tendency, at least in the first egg passages, to multiply most regularly and actively in the amniotic membrane as indicated by the constant and high concentration of complement-fixing antigen in this tissue as compared with other embryonic constituents. But after prolonged serial passage via the yolk sac and amnion or a more limited number of passages via the chorioallantoic sac,[20, 28] the virus becomes adapted to the allantoic system so that titers in the same range as those obtained with amniotic material may be anticipated.

VI. CERTAIN PROPERTIES OF THE EGG-ADAPTED VIRUS

A. Size as Measured by Filtration

In a series of experiments carried out in our laboratory,* the Elford technic of filtration through graded collodion membranes was employed to estimate particle size. The limiting pore diameter for infected allantoic fluid was determined as about 180 mμ, giving a range of particle size for the virus of mumps of 90 to 135 mμ These dimensions are of approximately the same magnitude as those determined for human and swine influenza viruses by this as well as by other technics[illegible] These viruses in certain other respects exhibit properties similar to those of the mumps virus[illegible] Habel,[illegible] employing infected yolk sac material in filtration experiments, obtained results which indicated a larger particle size. Possibly the higher value obtained by Habel was due to a choice of yolk sac material which tends to have a blocking action in filtration.

B. Thermoresistance

The infective and hemagglutinating properties of the virus are inactivated by heating for 20 minutes at 55° C. The activity of the complement-fixing antigen is destroyed by heating at 100° C. for 20 minutes but partially withstands heating at 80° C. for the same length of time. The factor eliciting the skin reaction is unimpaired by heating at 65° C for 20 minutes.

* Measurements were carried out by E Shlevin.

4. *Indications that infection has occurred.*

a. Death of embryo. The proportion of embryos which die following inoculation with a single strain of virus may vary over a fairly wide range. A considerable difference in the mortality rate has been observed when different lots of eggs are inoculated with portions of the same infective material. With the strain of virus employed in our laboratory, the death rate, following yolk sac inoculation of 6- to 7-day embryos, was after 7 to 9 days between 40 per cent and 50 per cent, on the average, although it ranged from 8 to 57 per cent in individual experiments. After inoculation of the amniotic sac of embryos of the same age, the mean death rate in various lots of eggs was about 25 per cent, with a range from 11 to 43 per cent. From the foregoing data, it is clear that whereas the virus is capable of killing the embryos, it does so irregularly, and therefore embryonic death cannot be regarded as a reliable index of infection.

b. Pathologic changes. There are no gross or microscopic pathologic lesions in either the dead or living infected embryonic tissues that can be specifically associated with the effect of the virus.

c. *Presence of hemagglutinin.* The demonstration of a specific hemagglutinin in materials derived from the inoculated egg may be regarded as a reliable index of infection,[21] but its absence may not necessarily indicate that infection has failed to occur. In certain instances, although this factor cannot be demonstrated, complement-fixing antigen has been shown to be present in the amniotic sac. The presence and concentration of hemagglutinin may be determined by the technic described in a subsequent section (VII, B, 2, a). Its specificity can be revealed by showing that hemagglutinative activity is inhibited upon the addition of specific antiserum to the system (Section VII, B, 2, b).

Employing hens' erythrocytes in the test, the highest concentration of hemagglutinin occurs in the amniotic fluid, even in material of the first egg passage, irrespective of whether the yolk sac or the amniotic sac has been selected as the route of inoculation. In contrast, the factor may not develop in the allantoic fluid under these conditions of inoculation until several serial passages have been carried out. Yolk fluid is also hemagglutinative according to Beveridge and his coworkers.[23]

Tests for hemagglutinin in amniotic membranes from earlier passages have yielded irregular results. Materials from later passages have

e. To distinguish other salivary gland diseases from mumps[25]

f. To detect the multiplication of virus in the parotid gland of the monkey that has been inoculated.[23]

g. To detect the multiplication of virus in the various tissues of embryonated eggs[20, 21, 23]

2 *Titration of antigens** A large number of tests have been carried out using the infected monkey parotid gland as antigen in the complement fixation test. This technic has been found to give regular, specific results A 20 per cent suspension of infected gland is prepared in saline by grinding with alundum It is then rotated at 3,500 r p.m for 30 minutes in the angle centrifuge, and the sediment is discarded The capacity of the supernatant to fix complement in the presence of either monkey or human serum known to contain antibody (convalescent serum) is determined by titration with 3 strongly positive sera, each diluted 1 8 The positive sera must give "2+ fixation" in a dilution of at least 1 128 with an antigen that has been previously standardized in the manner indicated immediately below In case the test is to be performed for the first time, an antigen diluted 1 30 may be arbitrarily employed to estimate in a preliminary manner the titers of the positive control sera, since a preparation of this sort in most instances contains sufficient antigen to allow strong fixation to occur with sera of even moderate or weak concentrations of antibody But the titer of antigen should be subsequently determined as soon as it has been found that sera with high antibody content are available The quantities of reagents the controls, and the procedure for carrying out the titration of antigen are the same as those described below for the complement fixation test itself (*see* Section VII, A, 6), except that serial dilutions of antigen are employed, whereas the concentration of serum is kept constant As controls for nonspecific fixation, 3 sera known not to contain antibody are included which are added to the same dilutions of antigen suspension The highest dilution in saline which gives complete fixation with at least 2 of the 3 positive sera is considered to be the end point A dilution containing twice this concentration of antigen is employed in routine testing of sera To control further the specificity of the reaction, a suspension of the parotid gland from a normal monkey is prepared in the same manner as the infected parotid, and is used in the same dilution as that of the infected parotid. This normal parotid gland control must always be included in each experiment If the antigen and control material are to be kept for a long period of time, they should be stored in the CO_2 cabinet It has been found practicable, however, to keep sterile undiluted material in the icebox for a period of 4 weeks, when tests are daily being performed

Since the monkey is an expensive animal, the embryonated hen's egg may be preferred as a source of antigen The amniotic fluid in all passages and the allantoic fluid, once the virus is adapted to the allantoic sac, are the materials of choice

* [illegible] com- [illegible] n the [illegible] more [illegible] well

C. PERSISTENCE ON STORAGE

Incomplete data on the persistence of the various activities of the virus under certain conditions of storage are available Infectivity persists for at least 12 months in the dry-ice box, for at least 8 weeks at 4° C, and for less than 96 hours at 23 to 25° C The hemagglutinin is probably somewhat more stable than the infective factor, but on the basis of existing information it can only be stated that it persists at least for 4½ months in the dry-ice box, 4 weeks at 4° C, and 7 days at 23 to 25°. The skin test antigen has been found to remain active after 6 months' storage at 4° C

D EFFECT OF CERTAIN CHEMICAL AND PHYSICAL AGENTS

Infectivity is destroyed by treatment with 0 2 per cent formalin within 24 hours at 4° C The hemagglutinin and complement-fixing antigen are not impaired by 0 2 per cent formalin within 4 weeks at 4° C At more elevated temperatures the hemagglutinin is impaired by the presence of small quantities of formalin *The infective capacity of the virus is removed by treatment with 1 5* volumes of ether for 30 minutes at 4° C Such treatment also reduces markedly the activity of the hemagglutinin, but the complement-fixing antigen remains undiminished under these conditions Exposure to intense ultraviolet radiation under the Oppenheimer-Levinson lamp brings about inactivation of infectivity *within 0 28 seconds The skin test antigen is not affected by the presence of 0 5* per cent phenol when maintained at 4° C within a period of 4 weeks

VII. DIAGNOSTIC PROCEDURES DEPENDING UPON THE DEMONSTRATION OF SPECIFIC ANTIBODY

A. COMPLEMENT FIXATION TEST*

1 *Applications* The complement fixation test has proved to be a useful tool in the laboratory. The following is a list of circumstances in which this test has been employed

a. For the determination of immunity in an individual exposed to mumps.[5]

b. To distinguish, from a group of potentially susceptible persons, the large number that would be resistant to the disease—of use in experiments for the evaluation of prophylactic agents, since about one third of the population has undergone an inapparent infection and is presumably immune [34]

c. To observe the effects of vaccination in man and monkey [25, 34, 35]

d. To diagnose mumps meningoencephalitis, particularly in the absence of involvement of the salivary glands.[9]

* The technic employed by Enders and his associates [18, 25] is described here in detail Others [20, 23] used a somewhat different procedure distinguished particularly by the fact that the complement is titrated in the presence of antigen, and fixation is allowed to proceed at 37° C for 1 hour.

cells and for fixation with antigen in the absence of antibody before it is used in a test.

5. *Hemolytic system* To 1 volume of a 2 per cent suspension of sheep's cells, washed 3 times in physiologic salt solution, 1 volume of diluted antisheep rabbit serum containing 2 units of amboceptor is added 15 minutes before the sensitized cells are used in the test.

6. *Procedure employed in the complement fixation test.* Appropriate 2-fold dilutions in saline of the inactivated serum to be tested are prepared in a volume of 0.5 ml., except for the lowest dilution, which should be prepared in a volume of 1.2 ml because of the many controls necessary. Usually these dilutions range from 1·2 to 1:128. Into a series of tubes, 0.1 ml. of each dilution is then pipetted and to each tube are added 2 units of complement in a volume of 0 3 ml., and then 0.1 ml. of appropriately diluted antigen The mixtures are shaken and kept overnight at 4° C. To each are added 0 25 ml of sensitized sheep's cells after all reagents have come to room temperature the following morning. The degree of hemolysis is recorded after incubation for ½ hour at 37° C. in the water bath. The end point is taken as the highest dilution giving definite fixation of complement denoted as 1+ Complete fixation is denoted as 4+.

The following controls are included in each test

1 Unknown serum alone (lowest dilution to be used in test)

2 Unknown serum (lowest dilution to be used in test) and 2 units of complement.

3 Unknown serum (lowest dilution to be used in test) and 1 unit of complement

4 Unknown serum (lowest dilution to be used in test), 2 units of complement, and normal parotid gland suspension

5 Two units of complement

6 One unit of complement

7 Normal parotid gland suspension and 2 units of complement

8 Normal parotid gland suspension and 1 0 unit of complement

9 Normal parotid gland suspension alone

10 Infected parotid gland suspension and 2 units of complement

11 Infected parotid gland suspension and 1 unit of complement

12 Infected parotid gland suspension alone

13 Salt solution

In addition a number of dilutions of a known positive serum are prepared sufficient to cover the end-point range To these are added infected parotid gland suspension and 2 units of complement Controls for the anticomplementary effect of this positive serum and its nonspecific reaction with the normal parotid gland suspension are included (*see* controls 2, 3, 4 above). A known negative serum

The amniotic membrane and the chorioallantoic membrane after the virus is adapted to it are found to possess high titers of complement-fixing antigens But with antigens derived from these sources a prozone occurs when small amounts of antibody are present, and they are, therefore, not as suitable as infected gland for routine testing of sera of low titer.[17] *The amniotic fluid or the allantoic fluid* may be titrated in the same manner as the monkey parotid gland suspension The average titers are low, that is, about 1.8, so that the fluid is usually diluted 1·4 or lower for use in routine testing of sera.

3. *Blood serum and other fluids* Human serum is heated at 60° C, monkey serum at 62° C., for 20 minutes just before the tests are carried out. If the serum is found to be anticomplementary, heating for a second time, after allowing the serum to stand at 4° C. or even room temperature for an hour, will usually remove this property without affecting the antibody content.[18] The second heating may also be carried out after the serum is diluted and stored in the icebox overnight

Other fluids such as spinal fluid and hydrocele fluid may be heated at 60° C for 20 minutes just before the test is performed to remove any anticomplementary activity as well as to inactivate any complement that may be present.

4. *Complement* Sera from at least 6 guinea pigs are collected, pooled, and stored in glass-sealed ampules in the CO_2 cabinet No diminution in complement titer has been observed under these conditions during a period of at least 2 months Titrations of complement, nevertheless, are performed each time the complement fixation test is done because of variation in susceptibility to lysis of different lots of sheep cells These titrations are set up in duplicate as a check, since errors might be introduced because of the necessity for measuring such small quantities All the complement for one test is diluted 1 10 and kept in the icebox until used at some time later in the day One ml of this diluted complement is then further diluted to 1 60, and volumes of 0 20 ml, 0 19 ml, 0 18 ml, 0 17 ml, 0 16 ml, 0 15 ml, 0 14 ml, 0 13 ml, 0 12 ml, and 0 11 ml are pipetted into a series of tubes. It is sometimes necessary to shift this range of volumes with different lots of complement. The volumes are each brought up to 0 5 ml by the addition of physiologic salt solutions,* and 0 25 ml of sensitized sheep cells (*see* 5 below) are added The contents of the tubes are mixed and incubated in the water bath at 37° C for 30 minutes The unit of complement is taken as the smallest amount that gives complete hemolysis in the titration If the concentration of complement in a particular pool of guinea pig serum is unusually low (1 unit => 0 22 ml of 1 60 dilution), then it is best to discard this lot For use in the test, the complement diluted 1 10 is diluted further so that 0.3 ml contains 2 units

When antigens derived from chick embryos are employed, it is best to titrate the complement in the presence of that quantity of antigen to be used in the test, since such materials are known to enhance the hemolytic effect of complement

Each lot of complement is checked for hemolytic effect on unsensitized sheep

* The physiologic salt solution should be prepared in a concentration of exactly 0 85 per cent for regular results

subsequently demonstrated.[6] This is especially true where involvement of the salivary glands is absent or minimal Since titers of complement-fixing antibody exceeding 1:64 (1:192 final dilution) are rarely found in the sera of normal individuals (in about 2 per cent), except in those who have been infected with the virus within the preceding year, titers higher than this can be regarded as presumptive evidence of recent infection.

In cases where it is desired to invoke the complement fixation test as a diagnostic aid, skin testing should not be done since it may frequently be followed by the formation of specific antibody or its increase if already present.[7]

B. Inhibition of Hemagglutination

1. *Applications.* So far, the inhibition of hemagglutination by serum containing specific antibody has been employed in the following circumstances:

a. To demonstrate specificity of the hemagglutination observed in the fluids of the infected egg Several viral agents[38, 39, 40, 41] as well as other factors[42, 43] can cause agglutination of erythrocytes indistinguishable from that brought about by the fluids from chick embryos infected with the mumps virus.

b To demonstrate specific antihemagglutinin for diagnostic purpose in persons recently convalescent from mumps

2 *Technic*

a Titration of hemagglutinin Either the method of Hirst[35] or of Salk[44] may be employed In the authors' laboratory the latter procedure with minor modifications has been adopted as routine. The infected fluid (amniotic or allantoic) is diluted in series of 2-fold increments, using tubes 8 mm. in diameter with rounded bottoms. To 0 5 ml. of each dilution is added an equal volume of 0 25 per cent suspension of hen's cells, washed 4 times in physiologic salt solution. Human cells from Group O or from the rhesus monkey may also be employed The mixture is allowed to stand at room temperature for 1½ to 2 hours, until the cells have all settled Agglutination is characterized by a thin even layer of cells which covers the entire bottom of the tube Absence of agglutination is distinguished by the formation of a dense "button" of sedimented cells occupying a small area at the center of the bottom of the tube. Partial agglutination is indicated by a "button" surrounded by a narrow thin margin of cells Two con-

diluted 1:2, infected parotid gland suspension, and 2 units of complement with the usual controls for this serum should also be included

After the addition of saline to bring the volumes to equivalence with those of the test, the control tubes are maintained under the same conditions and each receive 0.25 ml. of sensitized sheep's cells the following morning.

If infected amniotic or allantoic fluid is employed as antigen, it is substituted in place of the infected parotid gland, and the corresponding fluid from normal egg is used as control in place of the normal parotid. The technic is otherwise identical with that described above.

7. *Interpretation* a. Complement-fixing antibody as an index of immunity. The available data indicate that when this antibody is present in human sera it has arisen as the result of previous apparent or inapparent infection except in certain persons who may have received mumps vaccine or the skin test antigen. Moreover, there is no evidence that in other acute infections the antibody may appear or increase as a result of an anamnestic response From the results of many hundreds of tests in which monkey gland was used as antigen, it would appear that even a very low concentration of antibody, as indicated by a weak fixation test (1+ in 1·2 dilution, 1:6 final dilution, of serum) may be interpreted as denoting a previous infection. Hence, because of the solid and enduring immunity in mumps, such a reaction may be taken to signify that the individual is immune.[6] Conversely, however, failure to demonstrate complement-fixing antibody does not necessarily exclude the possibility of previous infection since it has been found that 20 to 23 per cent of those giving a positive history of mumps may fail to give positive complement fixation tests.

b. Complement fixation test as a diagnostic procedure. Conclusive serologic evidence for the nature of an infection suspected to be due to the virus of mumps can be obtained by demonstrating an increase in antibody concentration of 4 times or more, during the course of the disease.[5] In epidemic parotitis, the antibody first appears in many individuals as early as the 5th to the 7th day after the appearance of glandular swelling and is to be found in nearly all cases by the end of the 2d week. Occasionally, antibody may be demonstrated as early as the 1st day following the emergence of symptoms Before the desirability of testing serum becomes evident in other conditions caused by the virus, particularly meningoencephalitis, the antibody may already have attained a high level, and no increase in titer can be

It was found that complement-fixing antibodies could not be demonstrated on the 10th day after infection, but were present on the 12th day and had reached maximal concentrations by the 14th day.[illegible] After 81 days the titers were found to have declined to levels characteristic of monkeys and men who in the past had suffered attacks of mumps. The antihemagglutinin, on the other hand, did not appear until at least 2 days after the complement-fixing antibody, and maximal concentration of antihemagglutinin was not recorded until 6 days after the complement-fixing antibody had reached its fastigium. Furthermore, on the 81st day after inoculation, antihemagglutinin was still present in high titer. These differences would appear to indicate that the antibodies involved in the two reactions are distinct and may each arise in response to a different antigenic element associated with the virus.

C. Virus Neutralization Test in the Embryonated Egg

Recently,[illegible] it has been shown that a virus-neutralizing antibody is present in the sera of convalescent monkeys and men as well as in concentrated gamma globulin solutions prepared from pools of normal adult human sera by the method of Cohn and his associates.[illegible] When the embryonated egg is used for the demonstration of this factor, the technic is basically the same as that applied to the titration of such antibodies resulting from other viral infections. A series of dilutions of the sera to be tested is prepared in which the concentration of serum is decreased 2-fold. To an aliquot of each serum dilution, an equal volume of diluted virus containing from 1 to 10 minimal infective doses for the embryo is added. The mixtures are allowed to stand at 37° C. for 1 hour when they are placed in the icebox until inoculated into groups of eggs. Either the allantoic sac or amniotic sac route may be employed. Each dilution of serum-virus mixture is injected into 4 to 6 eggs. After incubation for 5 to 7 days, pools of materials obtained from each group of eggs are tested, if the greatest accuracy is desired, by means of both the complement fixation test and the hemagglutination test. To save time and labor, however, tests for hemagglutinin may be run first on the amniotic or allantoic fluid from each embryo. The results so obtained may then be checked by complement fixation tests on the amniotic membranes from embryos inoculated with materials near the end point indicated by the hemagglutination tests.

Titers of neutralizing antibody in convalescent sera from men and monkeys have been reported to range from approximately 1:4 to 1:100. From Habel's data,[illegible] it would appear that although there is no quantitative correlation in convalescent monkeys between the amount of neutralizing antibody and that of the complement-fixing antibody, they are both present in nearly all cases.

VIII. INTRACUTANEOUS TEST FOR THE DETERMINATION OF RESISTANCE OR SUSCEPTIBILITY

This test has been shown to be of value in distinguishing persons resistant to mumps from those presumably susceptible to infection (*see* Section III and Reference 7).

trols are always included, one consisting of saline alone and cells and the other of normal egg fluid (amniotic or allantoic) and cells. The highest dilution of infected fluid giving complete agglutination is considered to contain 1 agglutinating unit One unit of virus is employed in the inhibition test. The unit of virus is determined each time the inhibition test is performed. Dilutions are recorded in terms of final concentration after the cell suspension is added.

b. Titration of antihemagglutinin. *The serum is inactivated at 56° C.* for 30 minutes Serial 2-fold dilutions ranging from 1:8 to 1:256 or higher are prepared in volume of 0.25 ml. To each is added 0.25 ml. of infected fluid diluted so as to contain 4 units of hemagglutinin. This procedure gives a final concentration of 1 hemagglutinin unit in the final mixture. The virus and serum are mixed, and then 0.5 ml. of 0.25 per cent suspension of hen's cells is added. After mixing thoroughly, the preparations are allowed to stand at room temperature for 1½ to 2 hours. The readings are made when the cells have completely settled. The tubes are viewed from the bottom of the rack, and the highest dilution of serum that completely inhibits the hemagglutination by virus is taken as the end point. The results are expressed in terms of the final dilution after all the reagents have been added. It is necessary always to include a specimen of serum from the patient taken during the acute stage as well as one obtained during convalescence, in order to demonstrate a specific increase in antihemagglutinin. A known positive and known negative serum should be titrated at the same time and in the same manner A virus control (1 unit of hemagglutinin and cells) should be included as well as a control consisting of only salt solution and cells, and one of only the unknown serum (lowest dilution) and cells.

3. *Interpretation.* The specific nature of hemagglutinin in the fluids of infected eggs is revealed when its activity is inhibited by mumps convalescent serum obtained either from the monkey or man and to a degree significantly less by the acute serum from the same subject. Thus when the test is employed as a diagnostic aid, a rise in titer of antihemagglutinin of at least 4-fold during the course of the disease may be interpreted as evidence that the infection was due to the mumps virus[21, 26] It is not, however, so satisfactory as the complement fixation test because acute or "normal" sera frequently exhibit considerable nonspecific inhibition.

4. *Comparison of results obtained by complement fixation test and test for antihemagglutinin.* In a study of sera from monkeys infected with mumps virus,

In the preparation of materials used in skin testing, it is best to use glands derived from monkeys that have been shown to be tuberculin negative according to the method described by Kennard and his associates[46]

B. TECHNIC OF INOCULATION

The test for dermal hypersensitivity to the virus is carried out by injecting 0.1 ml. of the skin test antigen intradermally into the mid-flexor surface of the right arm and 0.1 ml of the control material into the same area of the left arm. The sites of injection are examined after the lapse of approximately 48 hours. The extent of any erythematous reaction which may have occurred is measured in centimeters, and note is taken of any thickening or induration extending from the point of inoculation.

C. CHARACTER OF THE REACTION

If the individual is hypersensitive to the skin test antigen prepared from infected monkey gland, an area of erythema varying from about 1 cm to 6 or 8 cm. may be observed. The average size of the reaction is about 2 cm in diameter. Its maximal intensity is usually attained between the 24th and 48th hour after injection After 48 hours, the erythema rapidly fades. In most instances, at the height of the reaction the redness is either moderate or marked, and the margins of the involved area are clearly defined, occasionally the reaction may be so mild as to leave some uncertainty as to its precise dimensions, but careful inspection nearly always enables one to determine its extent with reasonable accuracy In the large majority of cases exhibiting erythematous responses, a varying degree of induration of the skin is observed In a small proportion, however, it may not be recognizable, though the redness may be pronounced Erythematous reactions without induration have been interpreted, however, as indicating the existence of hypersensitivity

When monkey materials have been employed, the incidence of nonspecific reactions at the site of the inoculation of the control material has been low (i e , 4 per cent) In only two instances among 288 tests was the control reaction sufficiently intense to render the interpretation of the test doubtful Control reactions in children and adolescents are even less frequent

Nonspecific reactions appear to be more frequent when egg materials are used, although the data on this point are not sufficiently extensive to warrant an accurate comparison with preparations of parotid gland.

Various tissues and fluids obtained from both the infected monkey and the embryonated egg have been shown to induce a mild inflammatory reaction in the skin of persons who have previously been infected with the virus,[6, 7, 19, 20] whether in the form of a recognized attack or a subclinical infection.

A. PREPARATION OF THE MATERIAL

The procedure is essentially the same whether infected monkey parotid gland or infected amniotic sac, amniotic fluid, or allantoic fluid be employed. These materials are collected under the same conditions as the antigens for the complement fixation test. When infected gland is selected, the pH of the suspension, consisting of 1 part of the tissue in 19 parts of physiologic salt solution, is usually found to be approximately 6 8 to 6 9 If the reaction is more alkaline, sufficient 1/10 N hydrochloric acid is cautiously added to bring the reaction to about pH 6.8, but in no event lower The material is then heated for 20 minutes at 65° C. A large floccular precipitate is formed and is removed by centrifugation at 3,500 r p m in an angle centrifuge for 30 minutes This manipulation serves not only to remove the gross aggregates of the precipitate but also to render the fluid free from smaller particles which may be present The slightly opalescent fluid is then diluted to a final concentration of 1 30 (basing the calculation on the original content of tissue) with salt solution containing sufficient phenol to bring the final concentration of the phenol to 0 5 per cent The material is transferred to small sterile vials and stoppered The usual tests for bacteriologic sterility under aerobic and anaerobic conditions are carried out, using inocula of at least 0 1 ml of the crude saline suspension before heating and of samples drawn from each of the small vials of the final product As an additional control, 0 2 ml of the bulk supernatant fluid is injected subcutaneously into each of 2 mice which are kept under observation for 10 days and should remain healthy during this period

When materials from infected eggs are employed as a source of antigen, they are treated in exactly the same manner Although a precipitate is obtained after heating amniotic sac suspensions, little or none appears in the fluids

No accurate methods for estimating the potency of various lots of skin test antigen have as yet been devised To provide, however, some criterion whereby at least roughly uniform preparations may be obtained, the skin test antigen is diluted with sufficient salt solution before injection so that the final concentration of complement-fixing antigen is equivalent to 4 times the smallest quantity required to give complete fixation of complement as determined in titrations carried out on starting materials It is realized that this method of standardization is largely empirical since it has not yet been determined whether or not the same substance is responsible for both complement fixation and skin reactivity

As control materials, glands of normal monkeys or the tissues or fluids of normal eggs maintained under the same conditions as the infected ones are prepared in exactly the same manner and are employed in the same concentration as the antigen

6. [illegible]

7. [illegible]

8. [illegible]

9. [illegible]

10. [illegible]

11. [illegible]

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D. INTERPRETATION

On the basis of a recent study,[7] it was concluded that the readings of the test may be interpreted as follows. Persons exhibiting erythematous dermal reactions exceeding 10 mm. in mean diameter 48 hours after the inoculation of inactivated virus obtained from the parotid gland of the infected monkey (positive reaction) may be regarded, from the practical standpoint as resistant to mumps. According to this criterion, an error of interpretation of the positive test of about 10 per cent may be made. This error would be reduced to approximately 2 per cent if a reaction larger than 15 mm. is taken as the criterion for the resistant state.

When an individual fails to react or exhibits a reaction of 10 mm. or less (negative reaction) it may be construed as a presumptive indication of susceptibility. It is difficult to give any exact estimate of the chances that any individual with a negative test will develop clinical mumps following exposure. It can be stated, however, that the average attack rate in 340 negative reactors included in 11 groups exposed to mumps was 28 per cent. The attack rates among the individual groups, however, varied considerably from this mean.

As yet there are not sufficient data available from which to determine whether or not these same criteria of interpretation may, from the quantitative point of view, be applied to the results of tests done with egg materials.

In conclusion, it should be emphasized that inoculation of skin test antigens gives rise to complement-fixing antibody or its increase in almost all individuals exhibiting positive skin reaction and in nearly one-half of those exhibiting negative reactions These facts should be taken into account when complement fixation is employed as a diagnostic aid.[7]

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POLIOMYELITIS*

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I. INTRODUCTION

POLIOMYELITIS (*infantile paralysis*) *is a common acute infectious* disease more often seen in children than in adults, which offers serious problems to the public health officer. It is prone to appear in epidemic form, particularly in the summertime. Clinically it is characterized *by sudden onset, with a short period of fever, headache, and* vomiting, and this is the extent of the clinical picture in the great majority of cases On the other hand, in an appreciable fraction of the cases, clinical evidence of myelitis occurs, often resulting in flaccid *paralysis of various groups of muscles.*

A CLINICAL CONSIDERATIONS

In the abortive or mild case, sometimes spoken of as a "minor illness," the onset is sudden, with fever, malaise, headache, vomiting, and perhaps sore throat—symptoms which may last from a few hours to a few days A pain in the back or a stiff neck are noted occasionally, but, in general, the physical signs are indefinite and brief, so that such cases escape recognition The frequency of these mild cases should be appreciated by clinicians, epidemiologists, and laboratory workers alike *From them poliomyelitis virus may often be isolated*

In the paralytic case, the onset and clinical picture for the first 2 or 3 days may be similar to those of the abortive type, but the disease usually lasts longer, with fever for 3 to 10 days, although generally not more than 6 In about half the paralytic cases, there are two bouts of fever, with myelitic symptoms confined to the second bout Early evidence of central nervous system involvement is indicated by stiff neck and stiff back as cardinal signs; and with it there may be restlessness, hyperactive reflexes, pain in the limbs, and, subsequently, muscle weaknesses The onset of paralysis may be sudden, and its spread may be rapid

The exact manner in which the virus of poliomyelitis *enters* the human body is unknown. It seldom gains access to the central nervous system solely and

directly by way of the nasal mucosa and olfactory bulbs But theoretically it may penetrate through a number of possible portals, including the mucosa of the oral cavity, the upper or lower gastro-intestinal tract, or conceivably the skin Once in the body, the virus shows affinity for three areas in particular (*a*) the intestinal tract (particularly the lower ileum) where it may survive for weeks; (*b*) the mouth or pharynx where it survives for a shorter period, and (*c*) certain areas of the central nervous system

But, regardless of how the virus enters the body, its major *known portals of exit* are the mouth and the anus The virus is probably present in these sites during the incubation period, but it seldom remains in the buccal cavity or pharynx longer than the first week of the disease[5,6] In the intestinal tract its detection during the first 2 or 3 weeks of convalescence is commonplace, and it may remain there until the 12th week from onset[4] or longer The extent of the clinical symptoms, or the degree of *paralysis* which a given patient shows, has little bearing on the amount of virus which may be present in the throat or the intestinal tract, for the mild abortive cases harbor the virus in these sites as readily as do the paralytic cases *In fact, as the symptoms may be so insignificant as* almost to reach the vanishing point, many so-called "healthy"[13] carriers may actually be convalescent carriers As a rule, mild cases and "healthy" carriers represent the same age group as that which one sees among paralytic patients, namely children varying in age from 3 to 18, and particularly children from age 6 to 12 * The part which mild cases or carriers, both convalescent and apparently healthy, may play in the general spread of the disease is unknown It is probably considerable and may account for much of the mystery which attends the inability to determine the manner in which poliomyelitis spreads

1 *Dissemination of the virus* may result from direct human contact, the most commonly accepted view, or possibly from contaminated objects, perhaps including food Extra-human hosts or "reservoirs" for this virus are unproved possibilities as far as epidemiologic significance is concerned

Summer weather and rural environments influence the prevalence and severity of poliomyelitis, in that epidemics are far more likely to occur in the summer and early fall than at other times of the year, and high case rates are prone to occur in suburban or rural surroundings, notably in children's summer camps

2 *The incubation period* is usually estimated at 10 days, but it may be as short as 4 or 5 days or as long as 35 days[4] Average figures place it at 10 to 12 for paralytic cases and 5 to 10 days for abortive cases, the difference being due, no doubt, to the fact that the prodromal symptoms in the paralytic cases may be so light as to escape notice It is good practice to date the onset of the disease from the first day of fever, even though the amount of the initial fever may be slight

B CHARACTERISTICS OF THE VIRUS

An outstanding property of the virus of poliomyelitis is its ability to give rise to experimental infectious myelitis in the monkey. It is

* Age groups vary in different parts of the world, and these figures apply to the northern half of the United States and to most of Europe.

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in the detection of characteristic lesions in the spinal cord that the most useful means of identifying poliomyelitis virus rests (*see* Section D 4b for a positive result).

Some of the other properties of this virus are more difficult to measure. Its size, estimated from filtration data at approximately 10 to 15 mμ marks it as one of the smallest filtrable viruses. In comparison with many bacteria, it is quite stable, remaining viable at icebox temperature in aqueous suspensions of feces for months, and similarly in pieces of infected spinal cord stored in 50 per cent glycerol for years. It can also survive in weak solutions of phenol and aqueous suspensions layered under ether, but it is readily destroyed by oxidizing agents such as hydrogen peroxide and potassium permanganate, by ultraviolet rays Heating for half an hour at a temperature of 50° to 60° C has been shown to inactivate the virus in aqueous suspensions. Much remains to be learned, however, about the thermal lability of this virus, especially when it is suspended in milk

Another characteristic of poliomyelitis virus is its rather narrow host range, for most strains are pathogenic only for monkeys and chimpanzees Some strains, like the Lansing strain,[5] however, will infect certain rodents, notably the Eastern cotton rat and mice A few other Lansing-like strains have been isolated, but apparently they are not common

C. THE DIAGNOSTIC LABORATORY

In a poliomyelitis virus laboratory most of the diagnostic measures available for the assistance of the epidemiologist or clinician are limited to those for detecting the virus There are as yet no practical immunologic tests which can be applied for purposes of clinical diagnosis. Furthermore, virus isolations are of value primarily to the epidemiologist and only secondarily to the clinician In fact, from the clinical standpoint, such measures are impractical so far as early diagnosis is concerned, for individual tests may require from 2 to 5 weeks for completion.

In any event, the laboratory which sets out to do this work should be prepared to approach the disease from an epidemiologic standpoint and should be equipped with special facilities and personnel The use of monkeys or primates is still essential, and these expensive animals require space, caging facilities, and care of a type which demands special planning, teamwork, and apparatus.

1. *Personnel.* The poliomyelitis diagnostic laboratory requires a team composed of at least three members.

A clinical member who can evaluate and carefully select the type of case or at least select the situation from which material to be tested can be chosen.

A member to be responsible for the preparation of inocula and for the study of experimentally infected animals and of other procedures associated with the experimental disease.

An animal man, of superior ability, who is responsible for the care of animals both before and after their inoculation, for the sterilization of cages; and for the taking of daily temperatures of inoculated animals He may also assist in autopsying the animals.

To this team one can add a technician to assist in technical procedures and to be responsible for the general over-all care of instruments, reagents, and glassware.

2. *Equipment and Reagents Commonly Used*

a Large dry-ice box (4 to 8 cu ft.) for preservation and storage of virus, or an electrically driven deep-freeze box
b Portable dry-ice container (Arctic hamper)* or large thermos jug
c Refrigerator—household model
d Lusteroid tubes, ampules, and small pyrex glass bottles with stoppers
e Ultraviolet light sterilizer for lusteroid tubes.
f Balance (500 gm to 0 1 gm)
g Centrifuge
  (1) Recommended, but expensive International refrigerated model PR-1 with assorted heads, including multispeed attachment for 18,000 r p.m
  (2) Small centrifuge for usual procedures (International clinical centrifuge).
h Standard supplies of sterile glassware (pipettes, petri dishes, flasks, centrifuge bottles, mortars, pestles, test tubes, etc), and sterile alundum or sand
i Syringes, needles, and trephines
j Best grade of glycerol for preservation of virus In order to make a 50 per cent solution it is mixed with either water, saline, or phosphate buffer
k Ten per cent formalin (neutralized) for fixing tissues
l Individual rectal thermometers for monkeys
m Old tuberculin for testing each new monkey
n Adequate supply of sterile cardboard containers, glass jars, and wide mouthed bottles for collecting specimens
o. Autopsy instruments, particularly heavy bone cutters

* Jewett Associates, Rochester, New York

p. Contamination trays and sterilizing equipment, including incinerator for destroying infected animals.
q Tattooing apparatus for use with monkeys
r Available facilities for obtaining histologic sections.
s Adequate laboratory clothing (gowns, gloves, etc) and facilities for their sterilization

3. *Risk.* Isolation of poliomyelitis virus from human sources as well as work with so-called human strains* of poliomyelitis virus is a dangerous business. The percentage of laboratory workers who have accidentally acquired poliomyelitis within the laboratory is appreciable. In the United States alone there have been reported, between the years 1941 and 1946 inclusive, two serious cases,[6, 7] one of them fatal, in both of which the infection was acquired in all probability in the laboratory.

For obvious reasons the use of sterile technics in handling infective materials is mandatory. Gowns and gloves are recommended. Persistent vigilance is necessary on the part of the director of the team if the individual members and the animal men are to follow the recommended sterile technics for an appreciable period of time.

D. Handling of Animals

1. *Primate quarters.*[8] The selection of the site of animal quarters is important In captivity, monkeys are noisy and dirty. This does not mean, however, that they should be installed in a remote dark corner where they will be readily neglected

A fairly large room is desirable within which individual cages to hold not more than 2 monkeys each may be placed within a general large cage. Individual cages may be placed free in a room provided that the windows are protected and that proper measures have been taken to prevent the ready escape of the animals into other parts of the laboratory Light, air, and cleanliness are highly desirable.

2. *Types of monkeys.* At present (1948) monkeys of various species are expensive as laboratory animals, and the supply of those imported for research purposes to this country is limited In view of growing demands it is necessary to use these animals with care, to guard the supply, and to budget their distribution. For this reason emphasis is placed in these directions on the usual sources and con-

* An arbitrary designation of the term "human strain" would include any strain of poliomyelitis virus originally isolated directly from a human source, which is in its early (first to fifth) monkey passages

ditions under which poliomyelitis virus has been isolated most frequently from clinical sources in the past. This has been done in order to avoid using these expensive animals on materials that might be regarded as unfavorable. Such directions apply here to diagnostic and not to purely investigative procedures.

The following species of monkeys have been most often used in poliomyelitis work:

Macaca mulatta the rhesus monkey, usually from India.
Macaca cynomolgus and/or *irus* or *mordax*: the cynomolgus (or Java) from the East Indies, Philippine Islands, or Malaya
Cercopithecus aethiops sabaeus and *griseoviridis*: the green African and grivet monkeys from West and East Africa
Cercopithecus aethiops centralis, the vervet monkey from West, Central, and South Africa
Cebus capucina the ringtail or capuchin from South America

Of this series the rhesus monkey has been most widely used and has been imported to this country by animal dealers in the largest quantities for this purpose. Various factors affect decisions regarding the use of any particular kind of animal Quite apart from their relative susceptibility to experimental infection by poliomyelitis virus, one should consider: (a) ease of handling, (b) size of animal if daily temperatures or bleedings are required, (c) susceptibility of animal to spontaneous infection (such as tuberculosis, dysentery, or parasitic infestations) as well as susceptibility to malnutrition and vitamin deficiencies, and (d) price By and large the rhesus monkey fulfills many of the qualifications for the best all-round animal for general use. Cynomolgus monkeys, however, have been considered by several to be more susceptible to infection with certain strains of poliomyelitis virus when given by certain routes

For practical purposes and for ease in handling, particularly with regard to taking rectal temperatures, it is advisable to use small monkeys in preference to large ones, the optimum weight being from 4 to 6 lbs There is apparently little evidence as yet available to indicate consistent differences in susceptibility between large and small monkeys

It is not good practice to use monkeys for more than one inoculation Conceivably there may be differences in resistance to poliomyelitis virus in monkeys, and those which survive an inoculation may be the resistant ones

On arrival in the laboratory, new monkeys fresh from the dealers

should be placed in special cages for observation. Those in poor condition should be isolated or eliminated, especially those with diarrhea, skin diseases, malnutrition, and cough. It is always advisable to observe monkeys for a period of about 10 days prior to their inoculation. During this period a certain amount of conditioning of the animal can be practiced, and those which are too far gone can be eliminated. Weak and sick animals suffering from malnutrition, diarrhea, etc., sometimes make a remarkable (but slow) recovery if properly isolated and given sulfonamide tablets and a good diet

3. *Tuberculin testing.* It is good and important practice to tuberculin-test all monkeys shortly after their arrival in the laboratory. This is done according to Schroeder's test, with 1 mg O.T., using the upper or lower eyelid as the site of inoculation.[9] Monkeys with positive evidences of tuberculosis should be eliminated immediately for at least three reasons: (*a*)they may die within 5 weeks, (*b*) they may spread the disease to other members of the monkey colony, and (*c*) they are a potential source of danger to laboratory personnel since they are often infected with human types of tubercle bacilli.

4. *Caging* The placing of a great many animals (that is, 5 or *more) in a large cage is not recommended. Opportunities for the* spread of disease can be reduced considerably if animals are kept in small numbers (not more than 2, if possible) in smaller cages—a useful size being about 36 inches by 36 inches by 30 inches (*see* Fig. 1). When kept in small cages, however, they should be let out and exer*cised daily. Monkeys confined within small cages for any length of* time without adequate exercise often develop stiffness and atrophy of the limbs—so-called cage paralysis. More than once this cage paralysis has been mistaken by inexperienced workers and others for experimental poliomyelitis

*Understandable objections have been raised to the practice of allow*ing inoculated monkeys to exercise in a common area between small cages, because of the possible chances of cross-infection between animals. This objection is justified in the case of cynomolgus monkeys and chimpanzees, for with the latter animal, spontaneous infection can be acquired in the laboratory But the fact remains that over a period of 30 some years of poliomyelitis research in which it has been common practice to allow more than one rhesus monkey per cage, spontaneous infections with poliomyelitis virus in this species seem to have occurred

with rarity.* There is, however, a notable exception to this statement.[10, 11] It is probable furthermore that the degree of isolation necessary should vary according to the route of inoculation used If virus is introduced intranasally, one should assume that some of this virus is swallowed and subsequently perhaps appears in the stools. Subcutaneous inoculation has also resulted in the appearance of virus in the stools of monkeys and chimpanzees.

5. *Care of monkeys* Monkeys are by nature dirty and their quarters will stink unless continued vigilance is used to avoid this unpleasant feature. Cleanliness of their quarters is imperative The trays at the bottom of individual cages should be removed daily and cleaned Individual small cages and their trays should be sterilized at the end of each experiment Cockroaches should be kept down in the monkey quarters by the application of a suitable spray. A program for the care of monkeys should take cognizance of the fact that someone should be present on Sundays and holidays In other words, monkeys should not be neglected, they require far more attention than do guinea pigs and rabbits, and the animal man or woman responsible for their care must be able to cope with this fact

a Diet The diet used in our laboratories consists mainly of crackers specially designed for feeding primates We have used Purina chow, but these small pellets fall through the false bottom of the cage where they are either lost or become contaminated with excreta. The "chimcracker"† developed by Dr. R. M Yerkes and associates,[12] because of its large size (approximately 3 by 2 by ½ inches) has proved more satisfactory ‡

Each monkey is fed as many crackers as it will eat per day (generally 2 or 3) Since chimcrackers do not contain adequate Vitamin C, it is important to supplement the diet by the addition on alternate days of 25 mg of ascorbic acid to the drinking water, to which a small amount of sugar is also added As an extra precaution, about once

* The Yale poliomyelitis laboratory has had a single instance of what might be termed unexplained poliomyelitis in its monkey colony during a period of 15 years

† Supplied by The Kennel Food Supply Co, Fairfield, Conn

‡ The chimcracker formula contains twelve ingredients and consists of 2d clear wheat flour, 40 8 per cent soybean meal, 16 5 per cent, cornmeal, 8 7 per cent, ground wheat 4 85 per cent, powdered skimmed milk, 4 85 per cent, ground raisins, 4 85 per cent bone meal, 4 85 per cent, salad oil, 4 85 per cent, molasses (black strap), 3 9 per cent, salt, 6 per cent wheat germ, 4 3 per cent, irradiated yeast, 1 0 per cent A sample analysis yielded the following proteins, 21 3 per cent fats, 6 9 per cent, carbohydrates, 52 1 per cent ash, 6 9 per cent, phosphorus, 0 98 per cent, calcium, 1 86 per cent and iron, 0 025 per cent

per week each animal receives some fresh fruit, generally an orange. Raw peanuts are given several times per week also.

Sick and malnourished animals should be fed the following each day: bread with milk and bananas, oranges, and carrots. If necessary, milk and eggs should be given by stomach tube, and fluid intake maintained by the administration of saline intraperitoneally.

b Marking of animals. Tattooing numbers on either the shaved head, forehead or chest (or other areas) is a useful way of labeling monkeys. One also performs a service to fellow research workers by using this method, for, in spite of all efforts, used laboratory animals find their way back to monkey dealers and will be resold presumably as unused animals unless they are marked with a characteristic tattoo number.

Another common method of labeling a monkey, which is far less satisfactory, is to place a collar and chain around the neck of the animal to which a metal tag is attached. Care should be exercised to see that the tag does not become detached and that the chain or collar does not injure the skin of the neck.

II. ISOLATION AND IDENTIFICATION OF POLIOMYELITIS VIRUS

A SOURCES OF MATERIAL

Poliomyelitis virus can be isolated by monkey inoculation from a number of human sources, but even if the most careful technic is followed, it is a common experience to encounter negative results. With each attempt to isolate virus from a human source, every chance for success should be taken One of the most useful rules is to rely on the fact that the chances of success are greater if the material for testing is obtained early in the disease (within the first 7 to 8 days), dating the onset of the disease from the first bout of fever, even though it be slight, and not from the onset of paralytic symptoms Frequently a patient, thought to be in the 7th or 9th day of the disease, turns out to be actually in the 10th or 12th day, after careful questioning has elicited the fact that he had an earlier bout of fever

A useful procedure when one is anxious to obtain a strain of poliomyelitis from the oropharynx is to visit the family of a hospitalized patient and to determine whether any other members of the family are in an earlier stage of a mild attack These may furnish more valuable specimens than does the patient in the hospital.

1. *Human autopsy material* from which the virus has been frequently isolated includes:

a. Spinal cord and medulla.

b. Pons and midbrain.

c. Intestinal contents and intestinal wall.

In removing central nervous system tissue at autopsy, it is well to have an assistant ready with sterile gloves and several sets of sterile instruments* or at least with the means for reboiling the same instruments frequently. The calvarium should first be removed, without cutting through the dura if possible. The assistant then proceeds with the removal of the brain, using sterile technic. If time or other circumstances do not permit the removal of the spinal cord, as much of the medulla as possible should be secured by using a long knife and reaching well down through the foramen magnum

When the spinal canal has been opened for the removal of the whole spinal cord, the procedure is to take out the cord without cutting longitudinally through the dura. The assistant can then slit the dura and place the pieces for testing in a sterile petri dish. A convenient size for these pieces is 1 cu. cm. Favored sites for virus are the medulla and cervical and lumbar sections of the cord. The cauda equina is not recommended as a source of virus Other appropriate sections of the cord should, of course, be placed in fixing solution for subsequent pathologic study.

2 *Clinical cases* Poliomyelitis virus has been isolated many times from:

a. Feces. *A 15 to 25* gm. specimen is desirable.

b. Pharyngeal (oro- or nasopharyngeal) washings (15 to 20 ml.)

c. Throat swabs (2 from each patient)

The commonest source and one which is easy of access is human fecal material This is because poliomyelitis virus seems to remain longer in this "site" than it does elsewhere within the body Thus poliomyelitis virus has been isolated from feces during the incubation period, the active disease, and convalescence Nevertheless the optimal time for collecting specimens is early in the disease, although, with exacting methods, positive results have been encountered up to the 12th week of the disease or even later This applies to both paralytic

and nonparalytic cases and asymptomatic carriers. Such carriers are often present in association (in both time and place) with known cases of poliomyelitis. How long the so-called "healthy" carrier state lasts is unknown, but it is probably similar to that of the convalescent carriers. At least no persistent carriers of either type have yet been detected.

The virus is not found in spinal fluid, and very rarely in the blood.

B. COLLECTION OF MATERIAL

1. *Autopsy material* should either be tested immediately, which is preferable, or be frozen and kept on dry ice in a refrigerating unit at -20° to -70° C., or be placed in a solution of 50 per cent glycerol and saline and kept in the refrigerator at 0° to 4° C. until tested.*

Some points with regard to the use of glycerol are: (1) do not put more than 4 or 5 small pieces of tissue in 50 ml.; (2) use only the purest and best brand; and (3) do not allow the tissue to remain untested any longer than is necessary. This last statement might be qualified with the comment that, as 50 per cent glycerol acts as a slow bactericidal agent, there is some virtue in allowing contaminated specimens to remain in it for a few days before they are inoculated.† Although poliomyelitis virus has been known to survive in 50 per cent glycerol for many years, it has also died out in this medium after a few months or even weeks

2. *Feces* The collection and shipment of specimens of feces require special directions If the patient is in a hospital, a sterile bedpan is used, and the specimen is transferred to the proper container with sterile tongue depressors If the patient, or suspected carrier, is at home, where a sterile bedpan or an appropriate substitute is not available, a practical method is to place several thicknesses of newspaper or sterile paper, brought for the purpose, on the bathroom floor and to instruct each candidate in turn to defecate onto the paper. The stool specimen is picked up with sterile tongue depressors, and the contaminated newspapers from each person are then properly disposed of. A 15 to 25 gm specimen is desirable; this is much larger than the specimen usually required for bacteriologic determinations As an in-

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* It is perhaps unnecessary to add that a bottle of glycerol should be available in any good pathology department, and the staff of the department should be well informed of its presence to avoid the embarrassing occurrence of having all the virus-bearing tissue from an autopsy inadvertently put in fixing solution

† The possibility of adding antibiotic agents to 50 per cent glycerol deserves to be investigated.

dividual container for a specimen of feces, a glass fruit jar is useful, but somewhat handier are unused cylindrical cardboard boxes of the type in which icecream is dispensed in drugstores (Fig 2).

3. *Pharyngeal (or nasopharyngeal) washings.*[35, 36] Various types of irrigating fluid such as sterile distilled water, broth, or 10 per cent heated horse serum† may be used to obtain nasopharyngeal washings from patients or suspected carriers of the virus. The irrigating fluid is introduced into the patient's mouth, either from a glass or through a large glass syringe. Patients are then encouraged to gargle the material back and forth into the glass or into a small sterile basin. The procedure is carried on over a period of at least 3 minutes The amount of washings is kept under 30 ml

4 *Throat swabs* Material is obtained by rubbing the oropharynx lightly with 2 sterile cotton swabs, which are immediately transferred to a test tube containing 1 to 2 ml. of sterile water or broth. This material can be used immediately for inoculation or can be kept frozen.

C. SHIPPING OF SPECIMENS

If frozen material is to be shipped short distances, it can be sent in a proper container, this may be a well-packed thermos flask containing dry ice If fluid materials are to be frozen and sent in this manner, lusteroid tubes or special small, heavy-walled pyrex bottles, such as those in which insulin or vaccines are dispensed, are useful as containers. Ordinary glass tubes that contain more than 1 ml. of fluid are liable to crack when frozen Special care in packing the thermos bottles is essential or the breakage may be high.

The necessity of keeping the specimens cool probably varies with the circumstances. For short distances requiring 18 hours or less, cooling is probably unnecessary. Actually, the survival time of poliomyelitis virus in human stools at room temperature is unknown, but the virus is known to remain viable for many weeks in this medium at icebox temperature (0–4° C.).

Autopsy specimens, stool specimens (small in amount), and the sediment from oropharyngeal washings and swabs may be sent at room temperature in 50 per cent glycerol. For these it is convenient to use small wide-mouthed bottles with tightly stoppered or capped orifices, and, for safety's sake, the top of each bottle may be wrapped with several layers of adhesive tape

† The value of heating horse serum at 56° for 45 minutes is to destroy possible "neutralizing substances" which might be present in the serum

The mailing case should be marked *Medical Specimen, Glass, Perishable, Rush, Keep in a Cool Place.*

D. MONKEY INOCULATION

One, 2, or 3 monkeys may be used for each specimen. It is considered conservative practice to use 1 animal.

1. *Preparation of inoculum from nervous tissue.* The material to be examined is weighed after being minced with sterile scissors and placed in a sterile petri dish. Areas that are more likely to yield virus include the medulla and the cervical and lumbar swelling of the spinal cord The weighed fragments of medulla or cord (1.0 to 1.5 gms. usually sufficient) are placed in a sterile mortar containing sterile sand or alundum, and this material is then ground with enough sterile water, not more than 1 or 2 ml at first, to make it into a fairly thick paste Grinding is usually done for at least 5 minutes (*see* Fig 3) Sufficient cold, sterile, distilled water is then added to make a 10 per cent suspension, and this is then transferred to a centrifuge tube where it is spun at low speed (2,000 r p m.) for 5 minutes. The supernatant fluid should be kept cool during this process, should be opalescent, and should contain no particles large enough to plug the lumen of the needle. It is advisable to make up from 10 to 15 ml. of suspension so that some of the material may be kept frozen for possible future use

a. Intracerebral inoculation The monkey to be inoculated should be properly marked in advance of the inoculation. It should be anesthetized, commonly with ether, the hair should be clipped away from the forehead and top of the head, and this area should then be shaved and marked The site of inoculation is the central area over the frontal lobe on the right or left side The skin over this area should be rubbed well with iodine and alcohol. Trephining of the skull can be accomplished by using a sharp instrument which will bore a hole 1 to 2 mm in diameter; 1 ml of the suspension to be tested should be inoculated intracerebrally. It is our practice to inject 1 ml or less, to a depth of about 1 cm *

If it is important to try to demonstrate virus by all possible means, the same monkey can be inoculated by other routes; 2 ml of the original suspension should be also instilled into the etherized animal's

* Howe and Bodian advise dividing the inoculum into two parts 0 5 ml , each part is then inoculated deep into the hypothalamus, 0 5 ml on the left and 0 5 ml on the right.[19]

nares, and this process repeated on subsequent days; also 5 to 15 ml. can be given intra-abdominally.

b. Intranasal inoculation. In carrying out the intranasal instillation, it is of value but not essential to have the monkey partly anesthetized with ether. The 2 ml. of inoculum is allowed to drop directly into each nostril from either a pipette or from a fairly large syringe with needle attached. During this process it is advisable to have the monkey held underneath a fixed glass plate by an assistant so that splattering of the infectious material can be controlled. Both of the operators should wear gowns and gloves for this procedure, and if the glass plate is not available, the operators should wear goggles.

2. *Preparation of inoculum from fecal material.* Mention has been made that relatively large (25 to 50 gm.) specimens are more desirable than small (1 to 5 gm.) specimens. In preparing this material for monkey inoculation, various procedures may be used, none of them is easy or foolproof, and any laboratory which is beginning to do work in this field may expect to encounter difficulties which may be overcome through a process of trial and error.

Primarily it may be desirable to divide the original specimen of feces in half, the second half being kept frozen or in the icebox for future use, in case the test is unsatisfactory, or in case there are other reasons to retest the specimen.

For fecal material, the intracerebral route of inoculation (alone or in combination with other routes) has been more successful in some hands than in others. There are various degrees of refinement in the preparation of the inoculum for this route, depending in some measure on the speed of the centrifuges used.* The danger of the intracerebral method is the ease with which certain stool extracts may give rise to a brain abscess in the inoculated monkey. As a substitute, the combined intra-abdominal (intraperitoneal) and intranasal inoculation is also recommended. For this reason two methods (a and b) are offered, one as a possible substitute for the other.

a. Intracerebral inoculation (included). A procedure recommended by the Virus Laboratory at the University of Michigan School of Public Health is as follows:†

(1) Inoculum. Grind at least 5 gms. of the specimen in saline with alundum.

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* A technic devised for the preparation of stool extracts through the use of the ultracentrifuge has been described and utilized by J. L. Melnick.[24]

† Personal communication from Dr. T. Francis, Jr.

5 to 10 gms. in 60 ml.
11 to 15 gms. in 100 ml.
16 to 20 gms. in 150 ml.

Transfer to 250 ml. bottle with size No. 6 rubber stopper, and shake for ½ hour. Let the suspension stand overnight in the refrigerator and then decant the supernatant fluid and centrifuge at 2,000 r.p m. for 30 minutes.

After centrifugation, the sediment is saved in a small flask for intranasal (i.n.) inoculation, and the supernatant fluid is transferred to a 250 ml. bottle to which 10 to 20 ml. of ether are added. The bottle should be tightly stoppered The suspension is then shaken daily for ½ hour for 6 to 7 days

Subsequently the suspension is centrifuged at 2,000 r.p.m. for ½ hour, and a sample should then be drawn out from below the ether layer and measured The ether is then evaporated, and this specimen is labeled i p for intra-abdominal (intraperitoneal) inoculation. This i p. specimen is then centrifuged at 4,500 r p m in a lusteroid tube for ½ hour, and from the supernatant fluid, at least 0 1 ml is cultured on a blood agar plate

Another sample of 10 ml is placed in a small lusteroid tube within the centrifuge cup in the icebox in order to chill the specimen It is then centrifuged at 4,500 r p m for ½ to 1 hour. From the supernate of this specimen, 3 ml are drawn off, and of this 0 1 ml. is cultured on a blood agar plate This specimen is set aside for use for intracerebral (i c.) inoculation.

The sediment is saved until the result of the culture is known, then returned to the i p specimen

(2) Inoculation Each animal receives:

0 5 to 1 0 ml. i c 1st day
10 ml i p. and 2 ml i n 3d to 4th day
0 5 to 1 0 ml i c 6th day (not regularly done)
10 ml i p. and 2 ml. i n 7th to 8th day
10 ml i p and 2 ml 11th to 12th day

b. Intranasal and intra-abdominal inoculation. The stool specimen is divided in half, and the second half is set aside for possible future needs. From the first half consisting of fecal material weighing about 10 gms., a 10 per cent suspension in cold, sterile, distilled water is made up in a tightly stoppered 250 ml. flask containing glass beads

with frequent shaking and allowed to settle in the cold. The supernatant fluid is then poured off into another stoppered flask, and it is again well shaken and allowed to settle. From the supernate of the second flask, the material is divided into two parts, I and II, generally amounting to between 20 and 25 ml. each.

Part I (20 ml., untreated with ether) is kept at icebox temperature for intranasal (i.n.) use. It is instilled into the nostrils (in 2 ml. amounts) of the monkey to be inoculated on 3 to 10 successive days; usually 7 days, according to the method of Howe and Bodian.[18]

Part II (25 ml) for the *intra-abdominal route* (i.p.) is immediately centrifuged at relatively low speed (15 minutes, 2,000 r.p.m.), and to the supernate, 15 per cent ether is added as a bactericidal agent. The etherized suspension is kept in a stoppered container in the refrigerator for 24 hours and then 0.1 ml. is cultured on a blood agar plate.

If the growth of bacteria (after 24 hours) is minimal or absent, the fluid below the layer of ether is removed and is then inoculated intra-abdominally, in amounts not exceeding 15 ml, into the same monkey in which the untreated fecal suspension (Part I) has been and is being daily instilled intranasally. The concentration of ether, 15 per cent, used in Part II is usually sufficient to destroy or diminish the number of bacteria in the suspension to permit its intra-abdominal injection without fear of inducing peritonitis. If bacteria persist, more ether may be added and the specimen kept for several days with frequent shaking of the flask. In spite of these precautions, in some instances, peritonitis unfortunately occurs.

3. *Nasopharyngeal washings.* The washings are transferred to a flask containing glass beads, the flask is tightly stoppered and shaken for 10 minutes. This suspension is subjected to light centrifugation, 2,000 r.p.m. for 10 minutes, 10 per cent ether is added to the supernate, and then the suspension is allowed to stand in the icebox overnight. On the following day, the etherized material is inoculated intracerebrally in 1 ml. amounts. The intracerebral inoculation may be supplemented by an intra-abdominal injection of 10 ml of the etherized suspension. The remainder of the etherized suspension may be either frozen or kept at icebox temperature for a second inoculation. Such re-inforcement is given as a 1 ml inoculum 5 to 7 days after the first inoculation if the animal has not shown signs of the experimental disease.

a. Throat swabs. The method of Howe, Wenner, Bodian, and

Maxcy [15] is recommended. Inocula are prepared by eluting the material from the cotton swabs in phosphate buffer at pH 8.* The eluate is then brought to pH 7 and treated with 20 per cent ether in the icebox (18 to 36 hours) at which time the ether is removed. The total amount of eluate should be small, not more than 1 ml. of original phosphate buffer solution being used. An inoculum of 0.9 ml. may then be injected intracerebrally into the test monkey.†

b. Other methods More delicate methods for the isolation of virus from human stools exist, and reference has already been made to the use of the ultracentrifuge in preparing material for intracerebral inoculation. This expensive and complicated instrument is not an essential part of the usual poliomyelitis laboratory's equipment.

4. *Observation of inoculated monkeys.* Monkeys inoculated with material suspected of containing poliomyelitis virus should be observed with care for a period of 30 days. It is good practice to examine and exercise these animals daily during this period. By this method early signs of experimental poliomyelitis, such as tremor, ataxia, and weakness of the limbs can be detected, and the animal can be sacrificed at an appropriate time if a strain of virus is desired The further value of daily examinations is based on the fact that early in the period following an intracerebral inoculation, one may get a traumatic, spastic paralysis, often manifest as a hemiplegia caused by an upper motor lesion resulting from damage due to the inoculation in the brain. It is important not to confuse this type of lesion with flaccid paralysis resulting from myelitis Temperature readings should be taken daily, preferably at the same time each day, using individual rectal thermometers which are sterilized in a strong disinfectant solution between use This precaution is recommended since thermometers may become contaminated with poliomyelitis virus, and tuberculosis has been transmitted from one monkey to another via the rectum [18]

The usual rectal temperature of rhesus monkeys varies from 102 2 to 103 5°, but temperatures up to 104° are not particularly abnormal The onset of the experimental disease (induced with human strains of virus), may follow an incubation period of from 4 to 25 days This is usually, but by no means always, heralded by a rise of temperature to 104° to 106° Fever is maintained from 1 to 6 days, during which

* Wenner has [illegible] ... concentration for recovering Lansing [illegible]

† With both [illegible] taining mucus and [illegible]

the development of other symptoms may appear quickly or slowly. These consist of ruffled fur, nervousness, tremors (often first noticeable as a fine tremor of the ears), ataxia, and finally weakness, to be followed by definite paralysis most easily detectable in the extremities, but it may involve the face, neck, or back. With the development of considerable paralysis there is usually a precipitous fall in temperature. (Fig. 4).

If and when the inoculated animal develops symptoms which are moderately or highly suggestive of experimental poliomyelitis, it should be sacrificed early so that the strain of virus may be available for passage or storage, and so that the diagnosis may be quickly confirmed by histologic examination. In most instances it may be wise to sacrifice the animal routinely at the end of the 30-day period of observation unless the evidence for a negative result is complete. It is not good practice to use the animal over again, at least for a test which involves the use of material different from that originally injected *

a Autopsy of animals An animal to be sacrificed can be killed by the injection of ether into the heart The carcass is then placed on several thicknesses of newspaper which can serve as a medium for the absorption of infected material, to be subsequently consigned to the incinerator Before incising the skin, the fur of the back and head of the animal should be swabbed down with lysol solution. The brain and cord are removed first, using two or three changes of sterile instruments It is useful to remove the cord with the dura intact, and then to open the dura with sterile scissors and forceps The spinal cord is then taken out, and several sections are taken from 3 levels (medulla, cervical, dorsal and lumbar) One of each of these is placed in fixing solution for eventual histologic study, and one is kept for passage or storage, either frozen or in 50 per cent gylcerol.

It is good practice to examine the thoracic and abdominal viscera for evidences of tuberculosis or other diseases The findings of visceral lesions may have distinct bearing on the monkey's symptoms and on the health of other animals in the monkey colony

b Criteria of positive result. The most important evidence of the experimental disease in the monkey lies in the histologic examination

* [illegible]

inoculation

of the spinal cord. Sections should be taken and examined from 3 or 4 levels—medulla, cervical, dorsal and lumbar. The lesions should be unequivocal before a positive diagnosis of the isolation of poliomyelitis virus is accepted. Such lesions are generally manifest in the gray matter of the spinal cord involving in particular the ganglion cells in the anterior horn. These lesions are likely to be most prominent in the cervical and lumbar regions. They pass through several stages, but at their height, and later, are characterized by destruction of neurons and neuronophagia, and, prominently, by perivascular and interstitial round cell infiltration In the brain, lesions are not likely to be extensive but may be widely scattered, often in the base of the brain and with some selectivity for the motor cortex (especially area 4) and the vestibular nuclei and cerebellar centers.

A positive diagnosis is unwarranted if the lesions are merely suggestive, or if lesions are found only in the medulla without extension into other levels of the cord. By adherence to this rule one will not readily confuse experimental poliomyelitis with other types of encephalomyelitis, or areas of inflammation surrounding a brain abscess or brain injury. In many instances it may be wise to pass the strain of virus to another monkey for confirmatory evidence, and under certain circumstances it may also be wise to test the strain intracerebrally in mice, rabbits, and guinea pigs If these animals are infected it can be anticipated that one is either not dealing with poliomyelitis virus, or is dealing with one of the unusual (Lansing-like) strains of poliomyelitis virus which can be adapted to rodents. This possibility can be quickly determined by testing the host range in various rodents, including the cotton rat.

A negative result should be recorded if the animal fails to show lesions in the spinal cord of the type requisite for a positive diagnosis. In general the failure on the part of the inoculated animal to develop any appropriate symptoms during the period of observation is a fair indication of a negative result, but nonparalytic and apparently nonsymptomatic experimental poliomyelitis may occur in a fair percentage of inoculated monkeys.

An incomplete or unsatisfactory result is recorded if the inoculated monkey dies from some cause other than poliomyelitis before the 30-day period of observation is complete; under these circumstances the test should be regarded as unsatisfactory and should be repeated.

E. COTTON RATS, MICE, AND OTHER RODENTS

These species are necessary in any poliomyelitis laboratory because, in the event that new strains or questionable strains are isolated, it is important to test their infectivity in those rodents which are susceptible to infection by Lansing-like strains of the virus.

The strains may be inoculated intracerebrally in the form of a 10 per cent brain suspension, using a dosage of 0 05 ml. for cotton rats and 0.03 ml. for mice.

F. IMMUNOLOGIC IDENTIFICATION OF POLIOMYELITIS VIRUS

At present there are no satisfactory immunologic tests available for the identification of poliomyelitis virus Also, there are no satisfactory methods of diagnosing human clinical or subclinical poliomyelitis through immunologic methods In this connection it is important to note that a variety of strains of poliomyelitis virus exist, and the ability to apply immunologic methods may not be practical until different strains have been better identified.

G TISSUE CULTURES AND EGGS

Several strains of spontaneous mouse poliomyelitis virus and poliomyelitis-like viruses have been grown in tissue cultures and in developing eggs.[19] As yet there are no completely substantiated reports of the growth of monkey pathogenic strains of virus in tissue cultures or eggs. These technics will therefore not be discussed here

H. PRESERVATION OF VIRUS

A time-honored and satisfactory method of storing poliomyelitis is to keep it at icebox temperature (0–4° C.) in the form of infected monkey cord in 50 per cent glycerol and saline sol. Although virus has been known to remain viable in this medium for years, it is unwise to count on this, and strains which one wishes to maintain should be passed every 6 or 8 months

If a dry-ice box is available the material may be kept frozen (in lusteroid or heavy glass tubes) in the form of small pieces of nervous tissue or 10 to 20 per cent suspensions of nervous tissue, feces, and nasopharyngeal washings The extremely low temperatures (–70°) recommended for some of the other viruses are probably unnecessary for the preservation of poliomyelitis virus, for it is probable that –20° C.

of the spinal cord. Sections should be taken and examined from 3 or 4 levels—medulla, cervical, dorsal and lumbar. The lesions should be unequivocal before a positive diagnosis of the isolation of poliomyelitis virus is accepted. Such lesions are generally manifest in the gray matter of the spinal cord involving in particular the ganglion cells in the anterior horn These lesions are likely to be most prominent in the cervical and lumbar regions. They pass through several stages, but at their height, and later, are characterized by destruction of neurons and neuronophagia, and, prominently, by perivascular and interstitial round cell infiltration. In the brain, lesions are not likely to be extensive but may be widely scattered, often in the base of the brain and with some selectivity for the motor cortex (especially area 4) and the vestibular nuclei and cerebellar centers.

A positive diagnosis is unwarranted if the lesions are merely suggestive, or if lesions are found only in the medulla without extension into other levels of the cord. By adherence to this rule one will not readily confuse experimental poliomyelitis with other types of encephalomyelitis, or areas of inflammation surrounding a brain abscess or brain injury. In many instances it may be wise to pass the strain of virus to another monkey for confirmatory evidence, and under certain circumstances it may also be wise to test the strain intracerebrally in mice, rabbits, and guinea pigs If these animals are infected it can be anticipated that one is either not dealing with poliomyelitis virus, or is dealing with one of the unusual (Lansing-like) strains of poliomyelitis virus which can be adapted to rodents. This possibility can be quickly determined by testing the host range in various rodents, including the cotton rat.

A negative result should be recorded if the animal fails to show lesions in the spinal cord of the type requisite for a positive diagnosis. In general the failure on the part of the inoculated animal to develop any appropriate symptoms during the period of observation is a fair indication of a negative result, but nonparalytic and apparently nonsymptomatic experimental poliomyelitis may occur in a fair percentage of inoculated monkeys

An incomplete or unsatisfactory result is recorded if the inoculated monkey dies from some cause other than poliomyelitis before the 30-day period of observation is complete, under these circumstances the test should be regarded as unsatisfactory and should be repeated.

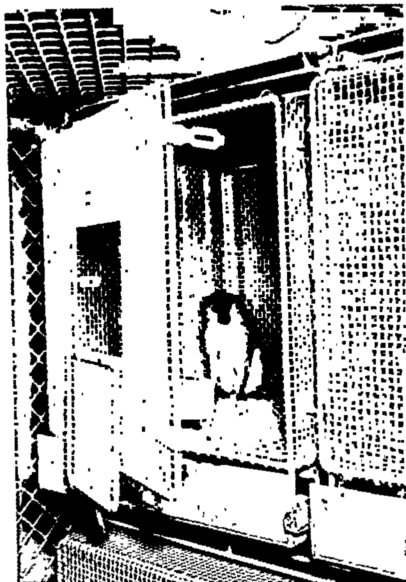


FIGURE 1 A convenient-sized monkey cage designed to hold one or two rhesus monkeys Dimensions of cage 36 inches by 36 inches by 30 inches fitted below with a sliding tray The animal in this cage is a green African monkey (*Cercopithecus aethiops sabaeus*)

is adequate. This allows the use of one of the less expensive electrically driven deep-freeze boxes for this purpose.[20]

Lyophilization of suspensions of spinal cord as a method of preserving poliomyelitis has been accomplished with various diluents such as (*a*) 10 per cent monkey sera and (*b*) mucin. Sufficient data are not available to say how efficient this method is for the preservation of *this virus*

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20 [illegible] s and of [illegible] *Dis*, 79 27–32, 1946

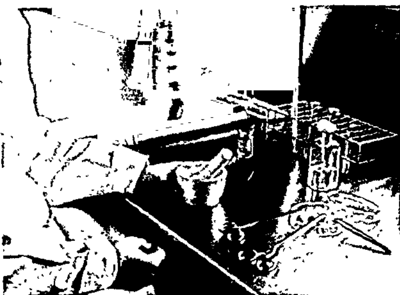


FIGURE 3 Instruments, apparatus, and reagents (50 per cent glycerol in screw-capped bottle and physiologic saline in bottle with cotton plug) used for preparation of a spinal cord suspension. The Waring blendor shown in the left background is a valuable adjunct for use in homogenizing stool suspensions, etc., but it is not recommended for general use unless it is equipped with a special sealed top



GURE 2 Types of containers useful for the collection and transportation of stool and other types of specimens The lusteroid test tubes with screw caps are particularly valuable for the collection of small (not larger than 10 ml) fluid specimens which are to be kept frozen The wide-mouthed bottle with the screw cap is also useful for storing materials in glycerol If it is used for transporting specimens, several thicknesses of adhesive tape should be wrapped around the base of the metal cap

ENCEPHALITIS

(Arthropod-borne Virus Encephalitides and Lymphocytic Choriomeningitis)

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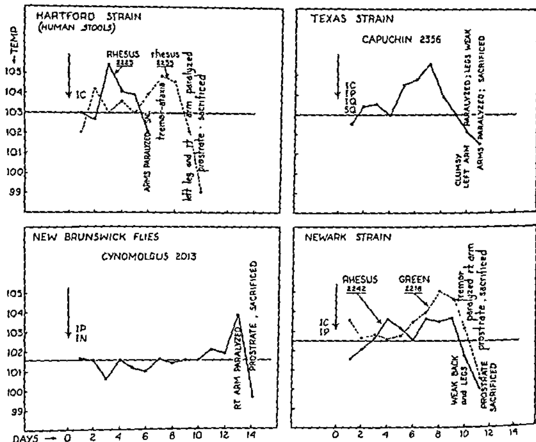


FIGURE 4 A series of temperature charts that indicate the course of 6 examples of experimental poliomyelitis induced by various routes with 4 different strains of virus in 4 species of monkeys The vertical arrows indicate the time of inoculation and the routes are designated as IC = intracerebral, IP = intra abdominal, IQ and SQ = intra- and subcutaneous, and IN = intranasal

In the upper left corner, the chart shows two cases of experimental poliomyelitis in two rhesus monkeys in which the inoculum was prepared for intracerebral injection by the use of the ultracentrifuge

serologic tests to be performed can be judiciously selected[8] if one knows what viruses are active in the area where exposure occurred, if the season is that of activity of the recognized vector (spring for ticks, summer for mosquitoes), and if exposure to bites is probable.

As a group, these viruses range in size from 19 to 60 $m\mu$ so are readily filtrable through the more common bacteriologic filters, and they will remain in suspension when centrifuged at speeds up to 16,000 or 18,000 r p m. Insofar as tests have been made none is adversely affected by the usual bactericidal or bacteriostatic concentrations of penicillin,[4] streptomycin,[5] the sulfonamides,[9] phenyl mercuric borate,[9] or 50 per cent neutral glycerol. Thus several procedures are available for rendering suspected tissue suspensions bacteriologically inactive for animal inoculation The viruses are inactivated by ether[10] and oxidizing agents and stabilized by certain reducing agents.[11, 12] The range of pH stability varies considerably from one virus to another.[12-15] All require 10 per cent or more of rabbit serum or other appropriate protein-stabilizing substance to preserve them for as long as 2 hours at 37° C. They tend to lose titer slowly over a period of a few days at 5° C. even in this medium In sealed tubes and in 50 per cent serum at the temperature maintained in dry-ice chests, a concentrated suspension will maintain its infectious titer for many months, and the viruses may be safely preserved thus for years, and possibly indefinitely without further passage These viruses may be lyophilized, and inactivation in the dried state proceeds very slowly even at room temperature. One of the most common, though less effective, means of storage is in 50 per cent neutral glycerol at 5° C.

II. METHODS OF ISOLATION AND IDENTIFICATION OF VIRUSES

A. PRECAUTIONS AND PREPARATION OF WORKERS

Several laboratory infections have occurred among those working with the Eastern, Western, and Venezuelan equine; with louping ill, Russian Far East, and lymphocytic choriomeningitis viruses. In most of these, the route of infection is not definitely known, but probably in the majority was by inhalation of finely dispersed virus particles. Under no circumstances should the Waring blendor or other similar mechanical device be used unless under an adequate hood. Previous to vaccination no one should be permitted to work with the Venezuelan virus itself or with the inoculated animals Vaccination[16] is also

I. INTRODUCTION

THERE is a large group of neurotropic virus infections of man, frequently encountered in epidemic form, with sharply delineated seasonal and geographical distribution. Certain of these can now be grouped as the *arthropod-borne virus encephalitides* since the viruses have been isolated from either mosquitoes, ticks, or mites, and convincing evidence has been presented to indicate that these invertebrate hosts are vectors, and occasionally reservoirs[1] Most of these virus infections are principally of vertebrates (mammalian or avian) other than man, the latter being a more or less accidental host. The following viruses can be assumed to belong to this group on present evidence: Western equine, Eastern equine, Venezuelan equine, St. Louis, Japanese B, Russian spring-summer Far East, and Russian spring-summer West (louping ill?). Probable members of this group of viruses, but less adequately studied are: *West Nile*,[2] *Bwamba fever*,[3] *Semliki forest*,[4] Ilhéus,[5] and California virus.[1] Lymphocytic choriomeningitis virus, although believed to be spread most frequently by excreta of rodents and to manifest a different epidemiologic and geographic pattern, is capable of experimental arthropod transmission. Since its diagnosis and clinical manifestations follow the general pattern of the others under consideration, it will be included in this discussion.

Most of the diseases in this group range in severity from the mildest type of illness, which gives no indication of primary involvement of the central nervous system, through a mild febrile meningitic form with spinal fluid changes and other clinical manifestations like those of nonparalytic poliomyelitis, to a severe central nervous sytem disorder characterized by stupor, tremors, convulsions, coma, and death.[6] The more severe cases are those usually recognized. Although these viruses are carried to man by blood-sucking arthropods (which generally acquire infection from the smaller vertebrates[1]), in some infections of man the stage of viremia is fleeting and possibly absent The same applies to the presence of virus in the spinal fluid In several types of these infections, serologic methods alone are available for laboratory diagnosis, unless the disease is fatal, which offers an opportunity of isolating the virus from the central nervous system tissue In many of the infections the pathologic lesions are so similar in pattern that the etiology cannot be determined by microscopic study alone. For details of the pathology of several diseases in the group the paper of Haymaker and Smadel is recommended[7] In general, the agent to be sought or the

usually indicated Storage at 5° C. to -10° C., obtainable in ordinary electric refrigerators or in a water tight container surrounded by H_2O ice is adequate for 1 or 2 days If longer storage is anticipated, the tissue or fluid should be placed in an adequately sealed tube and placed in carbon dioxide ice or other type of storage container maintained at -50° to -70° C. Penetration of carbon dioxide gas has been known to inactivate some of these viruses,[8] so that sealing against entrance of the gas under the greatly reduced pressure of an extremely low temperature is considered important If materials are to be shipped, the same criteria of temperature must be observed. Hemolysis of erythrocytes, due to freezing, does not appear to injure these viruses.[9] Frequently, portions of brain tissue placed in 50 per cent neutral buffered glycerol will yield virus if stored for several weeks at 5° C, and this method may be used to advantage if shipment is essential In no case should materials be left more than a few minutes at room temperature or above

D. Preparation of Materials for Inoculation

After sterility has been demonstrated, blood or spinal fluid may be inoculated without further preparation. Brain tissue should be suspended in 10 per cent inactivated* normal serum (preferably rabbit or other laboratory animal known to be free from antibodies) in saline The suspension can be prepared by thoroughly grinding for 5 to 10 minutes with a good abrasive such as alundum or pyrex glass chips or by mixing fot 2 to 4 minutes in a properly enclosed, screw-capped Waring blendor. Either a 10 or 20 per cent tissue suspension is usually prepared If this suspension is determined to be sterile, the supernatant may be inoculated after the suspension has settled in the icebox overnight, or after slow speed centrifugation for a few minutes The object is to sediment large tissue particles and abrasive. If contaminated, it must be treated further. Centrifugation at 16,000 to 18,000 r p m for 15 minutes in the angle head of an International multispeed attachment, surrounded by dry ice, is frequently effective.[10] Various antibiotic mixtures can be tried. One very effective combination is a final dilution of 1:10,000 of phenyl mercuric borate, streptomycin, 40 units per ml, and penicillin, 300 units per ml, left in contact at 5° C overnight This has been found to be satisfactory with Western equine, St Louis, and Japanese B encephalitis viruses[8] Probably less desirable is the use of a Berkefeld V filter, and least desirable, a Seitz filter, for although the size of these viruses permits passage, a large amount may be absorbed by the filter. When collodion membranes of large pore size (250 to 400 mμ) are available they are superior to the other more commonly used filters.

* 56° C for 30 minutes.

recommended for those working with Western equine virus. These two vaccines and the Eastern equine type can be obtained from certain manufacturers of biologicals as formalinized chick embryo suspensions prepared for horses or prepared especially for human use. No vaccine is available for lymphocytic choriomeningitis, but it is strongly recommended that only those known to have a significant titer of "accidentally" or normally acquired antibodies be permitted to work with it, where it is being used, or where infected animals are housed. Accidental infections with clinical manifestations with the St. Louis and Japanese B viruses have not been reported among the many workers in contact with them, so it may be assumed that with reasonable precautions, accidents will be rare.

B. SOURCES OF MATERIALS

In lymphocytic choriomeningitis infection, blood and spinal fluid should be tested for virus. These fluids have likewise been repeatedly reported positive in the Venezuelan equine, Russian spring-summer, and Japanese B diseases, but rarely or never with most of the others. Otherwise, gray matter, preferably selected from the brain stem, midbrain, and hippocampus, is selected for test.

C. COLLECTION, SHIPMENT, AND STORAGE OF MATERIALS

Blood and spinal fluid, if taken for test, should be collected only during the early, febrile phase Blood should preferably be heparinized, for virus may be adsorbed to cells [8] Citrate and oxalate should not be used because they produce convulsions when inoculated intracerebrally into experimental animals All fluids should be collected aseptically. Brain tissue, when possible, should also be obtained aseptically. Virus can most frequently be obtained from patients that have died during the first 5 days of illness, and when the autopsy is performed within 2 or 3 hours after death Enough isolations, however, have been made under less favorable circumstances to encourage other attempts if no better material is available Obviously, if embalming is performed prior to autopsy, the tissues are valueless for the tests to be described Selected tissues for *pathologic* study should be placed in 10 per cent formalin or Zenker's solution or both

In general, inoculation should not be performed until at least aerobic bacteriologic cultures are observed to be free from growth Thus, even though materials are delivered immediately to the virus laboratory, some storage is

pigs should be taken. The thermometer should be introduced to the 100° F. or the 38° C. line for a true reading, and only temperatures above 104° F. or 40° C. are of significance. Guinea pigs showing an elevation of temperature for 2 successive days should be sacrificed for passage of brain and spleen (the latter for lymphocytic choriomeningitis); otherwise, they are observed for 20 days unless signs are noted of weakness, tremor, salivation, inco-ordination, convulsions, or paralysis. Mice are observed up to 20 days for roughening of the coat, lack of or increased activity, or unusual behavior of any kind, tremors, circling, hunched position, stiffening of the tail, or paralysis. The "spin test," consisting of rotating the mouse one way, then the other while suspended by his tail, then watching his reaction after release, is frequently a valuable observation It may induce rolling or clonic or tonic convulsions. Any sick mouse is sacrificed for passage of the brain Eggs are observed for activity every hour or two between the 18th and 26th hours This is done in a dark room over a candler. Death of the embryo indicated by complete immobility with lack of pulsation and transparency of vessels is an indication for passage of *the embryo, if death occurs more than 18 hours after inoculation*

H. METHODS OF PASSAGE

Brains or spleens of animals or embryos from eggs are removed aseptically, and triturated in a 10 per cent suspension in the same manner as the original tissue was prepared. This suspension is cultured, sedimented, and stored at 5° to 10° C for 24 hours If bacteriologically sterile, the supernatant fluid is inoculated by the same routes previously employed in a group of the type of animal that has proved susceptible. After several successful serial passages the incubation period will usually decrease, inoculation can be performed by the intracerebral route only, and fewer animals need be inoculated (2 or 3).

A still more sensitive type of test is one employing serial blind passages of inoculated animals or eggs This method should only be attempted by those with experience and with the full realization of the many possible pitfalls.

I. PATHOLOGIC SPECIMENS, SMEARS, STAINS, AND FILTRATION

After it is certain that an infectious agent has been transmitted in serial passage, parts of the central nervous system tissue should be examined in stained microscopic sections and by impression smears

E. SELECTION OF ANIMALS

Mice are highly susceptible to all these viruses, and if only one kind of animal is to be used, the mouse is the animal of choice. Although any mouse will serve for certain viruses of the group, for satisfactory work with the St. Louis virus, the Webster neurotropic virus-susceptible strain of albino mouse (the Swiss-W) is preferred.[17] Chick embryos and guinea pigs are equally susceptible or more susceptible to certain strains of the equine viruses. Guinea pigs are more susceptible to some strains of the virus of lymphocytic choriomeningitis. There would appear to be no indication to include other animals in the primary attempt to isolate any virus of this group.

F. ROUTE OF INOCULATION AND AGE OF ANIMAL

Both guinea pigs and mice are most susceptible by intracerebral inoculation. With well-adapted strains of most viruses of the group, age of the animal has little effect on susceptibility, but this is not found to apply for certain freshly isolated strains. When available, mice of 6 to 8 days of age or younger are preferred. A group of 6 or 12 should be inoculated with .01 ml intracerebrally and 0 2 intraperitoneally and returned to their mothers for nursing. Unweaned guinea pigs may also be used with advantage, giving .05 ml. intracerebrally and 1.0 ml. intraperitoneally. If animals are used after weaning, mice may be given .03 ml. intracerebrally and 0 3 ml. intraperitoneally and guinea pigs 0.15 ml. and up to 5.0 ml., respectively Six or more animals should be used. Intranasal inoculation, in addition to the other routes, is not recommended. If eggs are employed, 6 or more of 9- to 12-day embryos are inoculated with 0.1 ml. by stabbing into the area near but not into the embryo (during visualization), or into the yolk sac, or if preferred onto the chorioallantoic membrane after dropping this membrane according to one of several methods devised for changing the position of the air sac. After sealing with wax or a paraffin-wax mixture they are incubated at 35° to 37° C.

G. TYPE AND DURATION OF OBSERVATION AND BEHAVIOR OF ANIMALS AND EGGS

Infant mice must be carefully observed several times a day for signs of illness, for should one become moribund or die it may be promptly eaten by the mother. Other animals are examined twice a day for appearance of signs of infection, and the rectal temperature of guinea

2. *Neutralization tests.* Specific hyperimmune sera to the more frequently encountered viruses are essential stock reagents in any virus laboratory. These are most conveniently prepared in rabbits. Injections are given subcutaneously or intraperitoneally beginning with a small dose, then proceeding with a series of increasing doses given once a week. The rabbit is bled after about 2 or 3 months. With the Venezuelan equine virus it is wise to begin with formolinized virus, then living virus can be safely used. No heterologous serum or other highly antigenic material should be incorporated in the inoculum

TABLE 2

Virus Identification by Neutralization Test. Results of Primary Virus Titration and Recommended Dilutions for Neutralization Tests

	Dilutions of Virus								
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Primary titration with normal serum	4/4*	4/4	4/4	4/4	4/4(L)	3/4	1/4	0/4(H)	0/4
Neutralization test proper									
Control serum				+†	+	+	+	+	
Serum A	+	+	+	+	+	+	+	+	
Serum B	+	+	+	+	+	+	+	+	
Serum C	+	+	+	+	+	+	+	+	

* Numerator indicates number of mice that died, denominator the number inoculated
† Indicates that this mixture of serum and virus is to be prepared and inoculated
The significance of (L) and (H) is discussed in detail in the text.

Before setting up a neutralization test it is usually economical to make a preliminary titration of a sample of frozen virus suspension Details are given in the section on neutralization tests, page 203. Three or more hyperimmune sera are selected among which the unknown is most likely to be represented. Table 2 illustrates the primary titration and the setup of the final test. For each of the hyperimmune sera and, for the control, normal rabbit serum, a series of tubes is prepared to receive serum and virus dilutions. For the control serum, one dilution of virus below (more concentrated than) that which should kill all mice ("Lowest" (L) dilution) is to be included, together with all dilutions above (less concentrated) through that which should kill none ("Highest" (H) dilution). For each of the hyperimmune sera the lowest dilution used in the series should be three or four 10-fold dilutions stronger than the first in the control series. All the dilu-

stained with Giemsa and a stain like Macchiavello's, to rule out other obligate tissue parasites Animal inoculation following passage through a Seitz or a Berkefeld V or N filter will establish the filtrability of the agent. Prior to filtration, the 10 per cent suspension in serum-saline should be clarified by centrifugation at moderate speed (3,500 to 15,000 r.p.m.).

J. IDENTIFICATION

1. *Animal host range* At this time it is usually in order to inoculate several species of laboratory animals with the agent, some individuals

TABLE 1

Comparative Susceptibility of Certain Laboratory Animals to a Group of Neurotropic Viruses (on Primary Isolation)

Virus	Mice 4 weeks or older		Guinea pigs 250 grams or more		Rabbits		Monkeys	Chick Embryos (Death)
	i c	i p	i c.	i p.	i c	i p.	i c.	
[illegible]								
Japanese B	++++	++	±	0	0	0	+++	±
L C M	++++	±	++++	+++	0	0	+	+
Theiler's virus (T O)	+	0	0	0	0	0	0	0

of each species by the intracerebral route, others by the intraperitoneal or subcutaneous routes. Those inoculated by the peripheral routes should receive serial 10-fold dilutions of the virus suspension up to 10^{-3} or 10^{-4}. The results of these trials will frequently give a clue to the identity of the agent. Table 1 is useful for identification of the viruses of this group recognized on the American continent. It includes also the Japanese B virus and Theiler's poliomyelitis virus of mice The latter is frequently inadvertently picked up and can usually be recognized fairly well by its limited host range and low titer It is to be noted in regard to all these viruses that freshly isolated strains occasionally show peculiar behavior regarding pathogenicity for certain hosts.[13]

Final identification must be made by immunologic tests.

2. *Neutralization tests.* Specific hyperimmune sera to the more frequently encountered viruses are essential stock reagents in any virus laboratory. These are most conveniently prepared in rabbits. Injections are given subcutaneously or intraperitoneally beginning with a small dose, then proceeding with a series of increasing doses given once a week. The rabbit is bled after about 2 or 3 months With the Venezuelan equine virus it is wise to begin with formolinized virus, then living virus can be safely used. No heterologous serum or other highly antigenic material should be incorporated in the inoculum.

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	Dilutions of Virus								
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Primary titration with normal serum	4/4*	4/4	4/4	4/4	4/4(L)	3/4	1/4	0/4(H)	0/4
Neutralization test proper									
Control serum				+†	+	+	+	+	
Serum A	+	+	+	+	+	+	+	+	
Serum B	+	+	+	+	+	+	+	+	
Serum C	+	+	+	+	+	+	+	+	

* Numerator indicates number of mice that died, denominator the number inoculated.
† Indicates that this mixture of serum and virus is to be prepared and inoculated. The significance of (L) and (H) is discussed in detail in the text.

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tions used with the control are also included (L minus 4 or 5 through H). At the end of the period of observation (about 15 days) the LD_{50} for each serum is computed by the method of Reed and Muench [19] (see Table 5). If the unknown virus is represented by a suitable hyperimmune serum, the LD_{50} for that one serum will be 3 or more dilutions lower than that of the control serum and that of the others In some instances (St. Louis, Japanese B, West Nile group) some overlapping is expected to occur.[2]

When a hyperimmune serum has been prepared against the newly isolated virus, it is wise to test its protection against several known viruses, the reverse of the test described above.

Identification should not be considered complete by the neutralization tests alone. Either cross-protection or the complement fixation test or both are indicated next.

3. *Cross-protection tests.* A series of suitable animals should be vaccinated with the virus selected on the basis of the above neutralization test. These may be mice or guinea pigs, if both animals are susceptible, or only mice if guinea pigs are not susceptible. For immunization, an amount of virus is selected which will kill few or none by the intraperitoneal route, or if the animals are very highly susceptible by this route a 10 per cent brain suspension of the virus may be inactivated by a final dilution of 0 2 per cent formalin (U.S.P.) and used as such for two inoculations, followed by one of the living virus. In most instances 2 weeks after the second inoculation of living virus by the intraperitoneal route, the animal will withstand 100 to 1,000 or more LD_{50} doses of the homologous virus by the intracerebral route.

While immunizing the test animals, similar normal ones are used for a preliminary titration to determine the LD_{50} of a frozen ampule of the unknown virus This time animals are inoculated with virus taken directly from the tubes in which the serial dilutions are prepared without further addition of rabbit serum and without incubation. A frozen ampule of a known (stock) strain of the selected virus is also titrated and the L and H dilution determined.

In the test proper, sets of controls are inoculated with dilutions L minus 1 through H of the unknown and known virus and sets of vaccinated animals with dilution L minus 3 or 4 through H of each (see Table 3). Vaccinated animals should show definite protection in approximately the same degree to each of the viruses (known and unknown) if the identification by neutralization test was correct.

Identification may be further confirmed by vaccination with the unknown virus and challenge inoculation with one or more known viruses. With certain viruses protection against intracerebral inoculation is very difficult to obtain and in such a case an animal must be used which is susceptible by a peripheral route[20] If the challenge is to

TABLE 3

Cross-Protection Experiment and Results of Preliminary Titrations and Recommended Dilution for Challenge Inoculations

	Dilutions of Virus									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Preliminary titrations										
Normal animals with unknown virus	–	–	–	4/4*	4/4(L)	3/4	2/4	0/4(H)	0/4	–
Normal animals with Virus A	–	–	–	–	–	4/4	4/4(L)	2/4	1/4	0/4(H)
Test proper										
Animals vaccinated with Virus A, inoc. with unknown		+†	+	+	+(L)	+	+	+(H)		
Animals vaccinated with Virus A inoc. with Virus A				+	+	+	+(L)	+	+	+(H)
Normal animals with unknown virus				+	+(L)	+	+	+(H)		
Normal animals with Virus A						+	+(L)	+	+	+(H)

* Numerator indicates number of mice that died, denominator the number inoculated

† + indicates that this mixture of serum and virus is to be prepared and inoculated

The significance of L and H is discussed in detail in the text

be by intraperitoneal inoculation, vaccination should be by some other peripheral route.

4 *Complement fixation.* From some viruses in early passage good complement-fixing antigens may be prepared by the method of Casals and Palacios[21] This antigen can be tested with a series of known specific hyperimmune sera and the virus quickly identified. The complement fixation test is described on page 213 It has been found, however, that many strains of freshly isolated virus, and occasionally well-adapted strains, do not produce satisfactory complement-fixing antigens, so that this method frequently fails. In all instances, a second immunologic test should be employed to confirm results.

III. SEROLOGIC TESTS FOR IDENTIFICATION OF DISEASE

A. INTRODUCTION

It has been pointed out above that for the group of virus diseases under discussion, serologic tests are of prime importance for arriving at a correct diagnosis However, the available methods have several serious disadvantages Outstanding examples are: (1) all diagnoses are made in retrospect, usually after recovery of the patient, and (2) it is essential to have a series of serum specimens (minimum two), one of which must be taken during the early acute phase [22] when the correct diagnosis may not have been considered, an omission that cannot be remedied later.

Two types of test are available, virus neutralization and complement fixation The former has been used extensively for many years Confidence in its specificity, knowledge of many of its shortcomings, and certain partially standardized procedures and modifications have kept it in the position of the favored test Newer knowledge, however, regarding the effect of temperature and of storage on certain antibodies has necessitated re-evaluation of comparative methods previously considered reliable.[23–25] At the time this is written the final answers to some of these vital questions are as yet undetermined For an excellent, broad, and up-to-date discussion of the theory and practice of the neutralization test, reference is made to Olitsky and Casals [26] The complement fixation test, by contrast, is a relatively new tool in this particular field Antigens, though greatly *improved by the extensive work of Casals,[21, 27, 28] are still relatively crude, vary* greatly in potency and specificity, and in some instances become anticomplementary after relatively short periods of storage [8, 29] Although a large amount of work has been done with hyperimmune animal sera [30, 31] there has been little opportunity to study the pattern of human response and only for a few of the viruses [8, 20, 28, 32–38] The antigen described today will quite possibly not be the one used 2 or 5 years from now, and better antigens may place the test on an entirely different relative footing Generalities about test results as expressed today will undoubtedly be extensively qualified after more experience It thus becomes obvious that insofar as both serologic tests are concerned this is not the time for recommending "standard procedures" Instead, a description will be offered of the tests now performed in one or more laboratories, accompanied by a discussion of some of the known pitfalls and limiting factors

One of the outstanding difficulties encountered in performing or interpreting *these tests relates to storage* and transportation of serum specimens The simpler types of comparative titration tests on paired sera described, apply only to sera stored and transported under almost ideal, intelligently planned, and closely supervised conditions Under less ideal, but more frequently encountered, circumstances these tests may be unsatisfactory or misleading, and it is proposed to point out where modifications of each test and certain other facts may be used to advantage. These more varied, less ideal circumstances of collection and storage

are those encountered by any state, army, or navy special diagnostic laboratory or by a university or other research center which offers its services in the diagnosis of this group of diseases Every one of these laboratories is faced repeatedly and most frequently with the practical problem of how to handle specimens from the individual case. The specimens are usually far from ideal in many respects, yet cannot in any way be changed in retrospect The physician wants a *diagnosis!* The problem in each instance is what test or tests are most suitable for this one peculiar set of circumstances At present no single "standard procedure" will suffice.

B. COLLECTION OF MATERIALS

Olitsky and Casals' recommendations [26] are as follows:

Serum· It is important to bleed persons for test serum after several hours of fasting (for example, in the morning before breakfast or before meals) and to employ sterile technic About 20 cc of blood is withdrawn, and after it is clotted by keeping it at room temperature for one-half hour, the blood is centrifuged at 2,500 revolutions per minute for twenty minutes Clear serum is then pipetted If a preservative is needed a 1 50 000 dilution of phenyl mercuric borate* can be added (or 1 2,500 borate stock, 1 drop to each 1 cc of serum, as an estimate) The serum is placed in a hard-glass ampule, which is sealed, if a test tube is used, it should be closed with a cork or rubber stopper and the top should be completely waxed A glass container should be filled only to one-third its volume to prevent bursting during freezing The container is kept at about minus 76° C by means of carbon dioxide snow until tested It should be sent to the designated laboratory by the fastest possible method, preferably in a vacuum jar holding solidified carbon dioxide If this refrigerant is not available the serum should be sent fluid, provided it can reach its destination within thirty-six hours

All these precautions are necessary The freezing of serum to maintain its antibody level and to standardize more than one sample for comparative tests from the same source, and obtaining clear, sterile serum to obviate a possible aggregation of virus particles (and thus a nonspecific reduction in titer), which cloudy, fatty or contaminated serum may bring about, and again to standardize more than one specimen from the same person

The first specimen should be taken as soon as a neurotropic virus infection is suspected, the sooner the better If taken early enough in infections with certain of the viruses, this sample will fail to neutralize, and in such an instance interpretation of a later positive is greatly facilitated The next specimen may be taken between the 15th and the 21st day This specimen is relatively unimportant, unless speed of diagnosis is considered essential, for most negatives at this early date cannot be considered significant, and titers may be at an equivocal level A specimen taken 30 to 60 days after onset is essential in the series, and the latter interval is preferable.

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the encephalitis viruses this is generally the albino mouse; however, for the Sabin B virus rabbits are used, and often for lymphocytic choriomeningitis the guinea pig is used. A second choice way is the injection of serum-virus mixtures into developing chick embryos (Burnet, Keogh and Lush[37]). As a rule the latter technic merely supplements the one employing animals or is used only for special purposes

When the mouse is the animal of choice one takes into account its strain and age A selectively inbred strain proved to be of high and uniform susceptibility, such as the Swiss W or the Rockefeller Institute strain, should be used; Casals and Schneider[38] have demonstrated that such animals may be essential for certain viruses The age of the mouse is also important It has been shown[39, 40] that an increasing resistance develops in growing animals to the peripheral (extraneural) injection of certain neurotropic viruses, although young and old animals are equally susceptible to the cerebral (neural) injection of the viruses (It has since been demonstrated that only with the Venezuelan and Russian Far East types of the encephalitis viruses this "peripheral resistance" does not develop in mice with age) To overcome this factor the intraperitoneal test for neutralization was devised (Olitsky and Harford[41]); this is generally a more sensitive test than the intracerebral. However, the latter is ordinarily used because the intraperitoneal method requires mice which are 12 to 15 days of age Again, the physiologic barriers to peripheral injection,[39, 40] which appear with age, may develop irregularly in some mice; therefore twice as many animals, or more (i e, instead of 5, 10 or more for each dilution), must be injected; and to provide mice of definite age (dated mice) a large, expensive organization is essential. Lennette and Koprowski[42] in elaborating the peripheral test have shown, moreover, that for the St Louis type encephalitis virus the injection of serum-virus mixtures subcutaneously into mice 3 days of age or intraperitoneally into those 8 days of age yields a still more sensitive result—several hundred thousand times greater neutralization capacity. For Eastern and Western equine and Japanese B encephalitis viruses and West Nile virus the intraperitoneal test may be used in mice aged up to 14 days, for Venezuelan equine virus, up to 200 days[43]

3. *Types of test.*

a. Classified as to whether serum or virus is diluted.

(1). Undiluted serum with serial virus dilutions. This is the most commonly applied test, the least beset by technical difficulties, and the easiest to standardize for comparative purposes. It cannot, however, be unreservedly approved, for the following reasons:

(a) It does not represent a true titration of serum antibodies, and results of quantitative comparisons do not always parallel those obtained by serial dilutions of serum[43a]

(b) For comparative titrations (essential for diagnosis in most instances), it can be recommended only for use with frozen sera.[25]

(2) Constant amount of virus with serial serum dilutions. This test has the advantages of giving a true serum titration, of requiring the least amount of serum of any type of test, and of being applicable

C. NEUTRALIZATION TESTS

1. *General discussion.* In general principle, the neutralization test is a method to demonstrate virus-inactivating substances contained in the serum. When a certain quantity of virus is mixed with a "normal" serum and the virus-containing mixture is found to be adequate to kill a laboratory animal, yet death fails to occur in another animal given a mixture containing the same quantity of virus with another serum, the latter serum is said to neutralize the virus. In practice several graded amounts of virus or serum are employed, and several animals are inoculated with each mixture; thus crude quantitation is effected.

The neutralization test, in addition to being a diagnostic tool, may show the result of inapparent infections, can be used in epidemiologic surveys, and is used for the identification of newly isolated viruses Although the serum of certain species of animals contains nonspecific antiviral substances, as far as is known virus neutralization in the serum of man is usually specific, in that it results from contact with the virus. There are certain antigenic overlappings recognized, and in the neurotropic group the St. Louis, Japanese B, and West Nile complex is an example.[2, 31] In such instances the degree of serum neutralization of the homologous virus is always greatest.

The labile factor mentioned above which changes with storage or increased temperature may be one of serious import when using serum that has not been constantly frozen Shipping a liquid serum during the encephalitis season (hot summer) may result in a marked change in its neutralizing capacity.[19, 23-25] Storage of an acute phase serum for *3 or 4 weeks at 5° C will render it incomparable to a later fresh* sample; that is, for test by the ordinary comparatively simple method employing serial dilutions of virus[23-25] Since the labile substance is largely lost by heating at 56° C. for 30 minutes,[23, 24] or diluted out between 1·5 and 1:10,[26] routine inactivation[24] or employment of serial serum dilutions can be substituted.[25] Each of these latter, however, has its disadvantages This laboratory has had no experience with the routine inactivation procedure,[24] thus, no attempt will be made to evaluate it.

2. *Animals used and routes of injection* These are described by Olitsky and Casals[26] as follows:

There are two choices of laboratory recipients for serum-virus mixtures, when the neutralizing capacity of a serum is to be tested first, the serum-virus mixtures are injected into an animal which is uniformly and highly susceptible. For

may be measured and placed readily in narrow-necked pharmaceutical ampules. These are then sealed, shell frozen by agitation in a CO_2-alcohol ice bath, and stored in a dry-ice chest.

b. Titration of virus. At the same or different times, two or three frozen ampules are titrated. Each titration is made under the exact conditions of the neutralization test. An adequate quantity of cold diluting fluid, 10 per cent inactivated rabbit serum-saline, is first prepared. Since each ampule is titrated separately and in a manner identical to the other, the description of only one is given. Into a set of sterile tubes standing in an ice-water bath, diluting fluid for a series of nine 10-fold dilutions is pipetted accurately. When this has been accomplished, and not before, one ampule of frozen virus is rapidly thawed by agitating in a 37° C. water bath. With a sterile *volumetric* pipette, calibrated to deliver 0.5 ml, this exact amount of virus (0.5 ml. of a 0.5×10^1-fold dilution* is delivered into the first tube, which should already contain exactly 4.5 ml. of diluting fluid The pipette is then discarded This dilution (0.5×10^2-fold) will finally represent a 10^2-fold dilution of mouse brain after an equal amount of the serum to be tested has been mixed with it With a sterile 1.0 ml. *volumetric* pipette, the contents of tube 1 are mixed and 1.0 ml delivered into 9.0 ml of diluting fluid in the next tube The pipette is discarded In a similar fashion all dilutions through to the 0.5×10^{10}-fold are prepared Next, six cotton-stoppered, short, wide-mouth tubes (from which syringes with 3/8" needles can be easily filled) are selected for serum-virus mixtures. 0.2 ml of the 0.5×10^5-fold dilution is placed in the first tube, 0.2 ml of the 0.5×10^6-fold into the second and so on through 0.5×10^{10}. To each, then, beginning at the highest dilution, is added 0.2 ml of normal inactivated rabbit serum. The final dilutions are now 10^5- through 10^{10}-fold. These stoppered tubes are then shaken well and placed in a 37° C water bath for exactly 2 hours, then transferred to an ice-water bath. Six mice are inoculated per serum-virus mixture This is done under light ether anesthesia, 0.3 ml in each, by the intracerebral route No antiseptic should be used on the mouse's head These mice are observed for an arbitrary number of days, depending on the incubation period and the pattern of deaths of the particular virus and mouse strain used Ten days is usually an adequate period of time to observe practically all

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* To avoid the complexity of reciprocals in expressing dilutions, insofar as is practical, all dilutions will be stated as so many "fold" Thus a 10^{-2} dilution becomes a 10^2-fold dilution and a 2×10^{-1} dilution is a 0.5×10^1-fold dilution (20%).

to unfrozen sera (labile factor diluted out). However, it possesses the following serious disadvantages:

(a). Since only one virus dilution is employed, it is technically difficult to work within the narrow range required.[25] The amount of virus used must not be excessive or inadequate.

(b). The end points of titrations are likely to be more drawn out and irregular than those with undiluted serum.[8] To minimize the latter, longer incubation of virus and serum is necessary (24 hours or more at 5° C.),[8] prolonging a test which at best requires patient waiting for results.

(c). The test is not readily subject to standardization for reference to any constant or index such as the neutralization index.

b. Classified as to route of inoculation.

(1). Intracerebral test. The customary route of inoculation for both methods given above is the intracerebral one This route affords a much less sensitive test for demonstrating low titers of antibody and is entirely inadequate for some viruses.[20] It frequently requires an *in vitro* incubation of virus and serum mixtures, whereas injection by a peripheral route does not.[41] It is, however, the only practical test for most laboratories, because mice which are weaned can be employed, and small age differences in the mice are irrelevant.

(2). Intraperitoneal test. As described above, this test has great advantages of sensitivity not afforded by the intracerebral method, but it is associated with disadvantages in regard to a suitable supply of infant mice, which most persons consider insuperable. Nevertheless, it is an extremely useful modification which must be used occasionally in every laboratory

4. *Technics.*

a Preparation of virus A group of approximately 10 inoculated mice are sacrificed after many or all show signs of infection. The brains are removed aseptically and are ground in a sterile mortar with abrasive or in a sterile Waring blendor, in cold 50 per cent inactivated* rabbit serum-saline or whole inactivated rabbit serum. This diluting fluid is added in such a quantity to make a 20 per cent suspension The suspension is centrifuged at 2,000 to 3,000 r p m. for at least 30 minutes, and the supernatant is then removed to another tube From this, about 0 8 ml is placed in each of a number of sterile 1 0 ml ampules. By use of a 10 ml. syringe and a 2-inch needle the amount

* Heated at 56° C. for 30 minutes

been used, as observed by the control titration, dilution 2 can possibly be considered significant. If too much was used, only occasionally would a significant positive be missed if up to a 5- or 10-fold increase over the expected was employed. Nevertheless, under the latter circumstances, the test is repeated with those sera giving suspicious results (one or more mice protected).

d. Comparative titrations of serum.

(1). With serial dilutions of virus. If the titration is to be made using the more common method, serial dilutions of virus with undiluted frozen serum, the following technic is used. No change in that to be described would be indicated should one prefer to inactivate all sera at 56° C[24] before test or should one elect to test all sera at a 1:5 or a 1:10 dilution.[25]

Serial dilutions are prepared as for the screen test or, in practice, the same set of dilutions is used, and some sera are set up for the screen test at the same time that the previously selected "positives" are titrated with their earlier paired serum or series of sera. The number of dilutions to be used for each serum is one to be determined by judgment. Every serum is tested against dilutions 1, 2, and 3 at least, and late sera may be tested against one or more lower dilutions, particularly if the equine viruses are being used. Not less than 6 mice should be used for each dilution in the titrations if significant comparisons are to be made. One may use 4 mice per dilution if, instead of 10-fold, 5-fold dilutions are employed. This latter method is the one of choice in this laboratory, but most workers prefer 10-fold dilutions, possibly for convenience in making calculations. The LD_{50} dilution for each series of serum-virus mixtures and also that of the control are computed to 1 decimal point. The logarithmic LD_{50} is expressed as the exponent of the reciprocal of the end point dilution. Thus, if the LD_{50} dilution is $10^{-8.2}$, the logarithmic expression of the LD_{50} is 8.2.*

The neutralization index of each serum (except those failing to protect against dilution 1) is obtained by subtracting its LD_{50} from that of the control and converting the difference to its antilog. Thus, if the computed LD_{50} dilution of the control is $10^{-8.1}$ (LD_{50} is 8.1) and that

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* [illegible]

deaths that will occur from an intracerebral inoculation of a properly adapted virus in 3- to 4-week-old mice of a suitable, uniformly susceptible strain. Deaths are recorded daily. All dying in a period less than that of the minimal incubation period (determined by experience for each virus) are discounted as dying from extraneous causes. The LD_{50} (50 per cent mortality end point) is computed by the method of Reed and Muench[19] (see Table 5). The results of the tests on the three ampules are combined or averaged Following this preliminary titration with a "normal" serum to determine the LD_{50}, all unknown sera can be tested with a minimal number of dilutions.

c. Screen test. If a series of sera are to be tested from several patients, it is usually advantageous, from the standpoint of economy, to test first only the final serum from each patient to determine whether antibodies are present or absent. If absent, it is unnecessary to test the earlier sera. If antibody is apparently present, this will be confirmed by repeating, and at this time, with all sera included, results of a comparative titration are obtained.

In general, for the screen test, two dilutions of virus are used, but the dilutions selected differ in various laboratories. Since in some virus infections antibodies are slow to form and in some individuals do not occur in high titer at any time, it is now the practice in this laboratory to use two dilutions, the higher containing 10 to 25 LD_{50} Dilutions are made in an identical manner to those used previously in the virus titration, except that the dilutions are started in such fashion that one (which will be called dilution 1) will represent between 10 and 25 LD_{50}. In this laboratory, for practical purposes, we name the dilutions below (more concentrated than) 1, in descending order, 2, 3, 4, etc , and those above (less concentrated) 1, in ascending order, -1, -2, and -3 (see Table 4). Dilutions 1 and 2 (10-25 and 100-250 LD_{50}) are used with each unknown serum for the screen test. At the same time, dilutions 1, -1, -2, and -3 are added to an equal amount of normal rabbit serum for the control titration. A control titration is made with every test. All tubes are incubated 2 hours at 37° C, chilled and inoculated, using 4 mice for each unknown serum-virus mixture and 6 for each of the control dilutions If the *control titration* indicates that the dilutions were properly made, any serum protecting at least 3 or 4 mice (out of 4) in dilution 1, is considered tentatively as positive and scheduled for titration with the earlier serum or sera from the same patient. If too little virus has

TABLE 5

Calculation of the LD_{50} Dilution and the Neutralization Index of a Patient's Serum (from Titration in Table 4)

CONTROL SERUM

Final dilution of virus	Dilution No.	Proportion dead	Number dead	Number survived	Accumulated tables: Died	Survived	Proportionate mortality	Per cent mortality
$10^{-7.4}$	−1	4/6	4	2	↑ 5	2 ↓	5/7	71 (B)
$10^{-8.4}$	−2	1/6	1	5	1	7	1/8	12 (A)

Compute as follows:

(1) $\dfrac{50\% - \%\text{ mortality of first dilution with} < 50\%\text{ mortality (A)}}{\%\text{ mortality of first dilution with} > 50\%\text{ mortality (B)} - \%\text{ mortality in (A)}}$ = Factor of proportionate distance (C)

$$\frac{50-12}{71-12} = .64\ (C)$$

(2) Log of reciprocal of first dilution with <50% mortality (A) 8 4

(subtract) Proportionate distance factor (C) x log of dilution factor (10) which is 1 .6

Control Serum LD_{50} = 7.8

PATIENT'S SERUM

Final dilution of virus	Dilution No	Proportion dead	Number dead	Number survived	Accumulated tables: Died	Survived	Proportionate mortality	Per cent mortality
$10^{-4.4}$	3	5/6	5	1	↑ 6	1 ↓	6/7	86 (B)
$10^{-5.4}$	2	1/6	1	5	1	6	1/7	14 (A)

(1) $$\frac{50-14}{86-14} = \frac{36}{72} = 5\ (C)$$

(2) 5 4 (A)

5 (C)

Patient's Serum LD_{50} = 4 9

To Compute Neutralization Index

LD_{50} of Control Serum 7.8

LD_{50} of Patient's Serum 4 9

[illegible]

Log of difference 1 4

Number of LD_{50} in Dilution 1 (antilog of 1 4) = 25

Since Dilution 1 was calculated to fall between 10 and 25 LD_{50}, this titration was performed within a satisfactory range (25LD_{50} used). Dilution 2 on the basis of this calculation was 250 LD_{50}

of the unknown serum $10^{-6.1}$ (LD_{50} is 6.1), their respective difference is 2.0 and the antilog or neutralization index 100. If the control LD_{50} is 7.9 and that of the unknown 7.4, the difference by subtraction is 0.5, the antilog of which (*neutralization index*) *is 3.2. Table 4 gives* an example of the titration of a serum and its control; Table 5 the calculation of the LD_{50} dilution and the neutralization index. Calculation of the number of LD_{50} doses actually occurring in dilution 1 in this experiment is also included. This amount is essential to know, in case sera were included for the screen test. It will be observed that the

TABLE 4

Titration of the LD_{50} Dilution of a Patient's Serum and Its Control (for Calculation of LD_{50} see Table 5)

Final dilution of virus	$10^{-1.4}$	$10^{-2.4}$	$10^{-3.4}$	$10^{-4.4}$	$10^{-5.4}$	$10^{-6.4}$*	$10^{-7.4}$	$10^{-8.4}$	$10^{-9.4}$
Dilution number	6	5	4	3	2	1	−1	−2	−3
Control rabbit serum	–	–	–	–	–	6/6†	4/6	1/6	0/6
Patient's serum	–	–	6/6	5/6	1/6	0/6	–	–	–

* This dilution was selected as dilution 1 on the basis of the previous titrations. It was calculated to contain between 10 and 25 LD_{50} doses. All other dilutions used here were calculated to be ascending and descending multiples of 10 from this. The lowest dilution was $10^{1.4}$-fold (2.5×10^1, for the antilog of .4 is 2.5).

This dilution was made from the frozen ampule containing 0.5×10^1-fold dilution of brain by adding 0.5 ml. of this virus suspension to 0.75 ml. of diluting fluid, resulting in a 1.25×10^1-fold dilution. Serial 10-fold dilutions were prepared from this through 1.25×10^8. When an equal amount of serum was added to each they became dilutions of 2.5×10^1-fold ($10^{-1.4}$) to 2.5×10^8 ($10^{-8.4}$). Except for this calculation required to make the first dilution of the series, the decimal in the exponent of the dilution does not further complicate the calculation of the LD_{50} or the neutralization index.

† The numerator indicates number of mice dead, the denominator the number inoculated.

test results (dilution 1) were within the required limits (10-25 LD_{50}). In experienced hands, the range of variation is usually well under 10-fold (one log).

All sera which fail to protect at least 50 per cent of the mice in dilution 1 are given the neutralization index of 1, no calculation is required.

(2). With serial dilution of sera. If this method is selected there is no change in the manner of handling the frozen virus, and the same method of preliminary titration and titration of the control serum is

of Lennette and Koprowski [12] be employed. Either serial dilutions of serum or virus may be used, and calculations are made in the same manner as described for the intracerebral tests.

5. *Interpretation.* Because of antigenic differences in strains of the same virus, marked differences in the degree of response and time of development of antibodies with different viruses, and the lability of antibody under certain storage conditions, it is impossible to set lower limits for a significant positive response.

TABLE 6

Average Human Immunologic Pattern in Certain Virus Infections

Virus	Type Test	Weeks after onset 0–1	1–2	2–3	3–4	7–8	15–16	1 yr	2 yrs.
Western equine	Neut.	++	++++	+++++	+++++	+++++	+++++	++	+
	C. F	0	±	+	++	+++	++	±	0
St. Louis	Neut.	0	±	+	++	+++	++++	++	+
	C F	±	+	+++	++++	++++	++++	+	±
Japanese B	Neut.	±*	±	±	+	++	+++	++	+
	C F	0	+	+++	++++	+++	+++	+	?
Lymphocytic choriomeningitis	Neut.	0	0	±	±	+	++	+	+
	C F	0	+	++	++	++	++	±	0

* Reported once on 3d day (Sabin[8])

In a St. Louis infection, a neutralization index of 20 (by intracerebral test) in a 20-day serum, using one strain of virus, might be highly significant, if the test result is adequately confirmed by repeat tests. On the other hand, a neutralization index of 20 in another patient (Western equine infection), with at least one strain of Western equine virus, in a serum taken 20 days after onset would usually be considered as of no significance. The interpretation of a change between an acute phase and a convalescent serum cannot be made from a table or by any rule of thumb. Entirely too many variables exist, some poorly understood. Interpretation requires experience with the strain of virus used, an understanding of the biologic variabilities and the technical errors of the test, knowledge of and frequently complete control over matters of temperature and time of storage of sera, and an understanding of the usual and extremes of human immunologic response to the virus infection in question. Several of these infections have distinctly individual immunologic patterns so that no generalization is permissible. Table 6 will give some helpful comparative data on four of these viruses. The complement fixation response is included in the same table. The author has had adequate experience to feel complete assurance of the general accuracy of the pattern only with the Western equine and St. Louis viruses. His experience with the Japanese B is

employed with one exception; incubation is at 5° C. for 24 hours instead of 37° C. for 2 hours. With the control serum, however, the LD_{50} will be essentially the same, using either incubation procedure.

*Sera are set up in 5-fold dilutions, all serum virus mixtures are in*cubated at 5° C. for 24 hours, and calculations are made in a quite different manner. It has been demonstrated that more time is required for *in vitro* union of virus and antibody when dilute serum is employed.[25, 12] Without inactivating the virus, 37° C. cannot be continued over 2 hours, but incubation in the cold for 24 hours is a practical compromise.

It is usually advisable to employ about 4 serum dilutions with an acute phase serum, and 6 with a convalescent. The number of days after onset when the serum was collected and the usual time for antibodies to form with the virus in question should be considered as factors influencing choice of dilutions For dilutions of each serum, wide-mouth short tubes are selected. In each of the first two of a series is placed 0.2 ml of the serum to be tested To each but the first is added 0.8 ml of 10 per cent serum-saline diluting fluid. After mixing the contents of the second tube, 0.2 ml is transferred to the third, etc, in series and 0.2 is finally discarded from the last Virus is added to these same tubes; 0.2 to the tube of undiluted serum and 0.8 to all others In this manner, a minimum of serum and glassware is required. Virus dilutions are prepared as in the other test If other sera are simultaneously tested by the serial dilution of virus method or by the screen test, the same dilutions suffice Only dilution 1 (between 10 and 25 LD_{50}) is employed except for the control titration with normal undiluted rabbit serum. This control series must extend from dilution 1 through –3

The LD_{50} of each serum is computed again by the method of Reed and Muench, now on the basis of serum dilutions This measure has no absolute value as it had in the previously described test for it depends entirely on the amount of virus present in the test dose However, two sera from the same patient tested simultaneously are readily compared. Comparison is expressed as the arithmetic quotient of the reciprocals of two LD_{50}'s For example, if the LD_{50} of the first serum is 1.25 and that of the second 1 625, there is a 25-fold increase. If that of the first is 1:10 and that of the convalescent specimen 1:32, the apparent change is 3 2-fold

(3). Intraperitoneal neutralization test For performing this test it is recommended that the technic of Olitsky and Harford[41] or that

The second problem is closely related to that just described. Only sera collected and stored under ideal circumstances can be used with many of the antigens now available; and many of these sera are troublesome. Even though all sera are routinely inactivated at 60° C. for 20 minutes to reduce nonspecific reactions (those that are Wassermann positive and those that are slightly anticomplementary), 25 to 50 per cent may be unsatisfactory until heated twice at 60° C.[44] or once at 65° C. for 20 minutes.[37] Unfortunately, prolonged heating markedly reduces the titer of St. Louis virus antibodies in some human sera.[43b] Another disadvantage of this test is that it requires much larger amounts of serum than does the neutralization test. In many instances this matter is one of real importance.

Despite these difficulties, this test, when performed with the utmost respect for detail, is of great value as a diagnostic tool When suitable sera are available, it is frequently much more satisfactory for making a clear-cut diagnosis of St. Louis, lymphocytic choriomeningitis, and probably Japanese B virus infection than is the neutralization test. Furthermore, by its use, the diagnosis can be made much earlier in these three diseases

Since the time of appearance and wane of neutralizing antibodies is entirely different from that of complement-fixing antibodies. (see Table 6), these two tests may show some interesting differences in results which, when understood, instead of being confusing are of great practical value. For example, complement-fixing antibodies for the Western equine virus usually disappear between 1 and 2 years after infection (or sooner); therefore, the presence of one type of antibody in the absence of the other may have considerable significance in epidemiologic or diagnostic studies If, in a case of St Louis infection, only one serum was available, and it was taken on the 12th to the 20th day, the *confirmed* presence of complement-fixing antibodies without neutralizing antibody could probably mean only one thing—infection with St. Louis virus with recent onset

2. *Preparation of antigens* Until very recently the antigen developed and described by Casals[45] has been that generally used, although the modification of Havens and his coworkers[46] has also been used These antigens were highly infectious and inactivation by radiation or by other means was recommended[47] Benzene extraction of antigens was then introduced by DeBoer and Cox.[48a] Extraction removed much of the fraction which reacted with Wassermann positive

much more limited, and the literature, except on the neutralization test, is equally or more limited His knowledge of *the pattern of lymphocytic choriomeningitis* antibodies is based largely on the literature.

The Eastern equine virus probably behaves much like the Western as far as the neutralization test is concerned.

One fact must be emphasized in interpretation In infections with the four viruses listed in Table 6 at least, no significance can be placed on any positive result with a single convalescent serum Mild missed cases are known to occur frequently, and in certain communities a considerable proportion of normal residents *have antibodies to one or several of these viruses*[32] *A definite rise in titer* between two suitably spaced sera must be obtained, and the significance of the rise must be evaluated on the basis of several facts, including the time of onset, day of collection of sera, and temperature of storage of the serum at all times These data must be available to the laboratory director if he is to interpret the results of the tests. If the physician attempts to interpret the tests, he must have as thorough an understanding of the laboratory procedures and behavior of the animals and virus strains as the laboratory director

A negative neutralization test result probably has more clear-cut significance than any other test result, at least when as little as 10 or 25 LD_{50} of virus is used (see Table 6) and the serum is taken 2 months or more after the onset of illness. Individual cases are on record where neutralizing antibodies have apparently failed to develop, but in most instances this one test has unchallenged significance.

Of inestimable value in interpretation, in many instances, are the results of the complement fixation tests performed on the same sera If there is any doubt, do both tests.

D. Complement Fixation Test

1. *General discussion.* The chief problem in complement fixation is to obtain a good antigen. To date, mouse brain has proved to be the best source for most viruses of the group. Exceptions are the amniotic sac of the chick embryo for Western equine virus[43b] and the guinea pig spleen for lymphocytic choriomeningitis[82] Because of the extra controls required for each different type of tissue, the advantages of using a single type are usually considered as outweighing the gain in extra sensitivity for any one virus that might be obtained by using a variety of tissues. It is customary and advisable to use 3 to 6 antigens simultaneously. The disadvantages of mouse brain are: (1) it reacts nonspecifically with certain sera (Wassermann positive and some others), (2) it tends to become anticomplementary during storage, and (3) its anticomplementary properties are potentially greater than manifested, in that with certain sera, which are of themselves not demonstrably anticomplementary, a synergistic reaction occurs resulting in false positives. These false positives are frequently difficult to detect

For preparation of lymphocytic choriomeningitis antigens from guinea pig spleens, the original procedure described by Smadel *et al.*[32a] is satisfactory: "Complement fixing antigen occurs in spleen, consolidated lung, liver and brain of acutely ill guinea pigs infected with the virus, but not in tissues from normal animals. The antigen is most readily obtainable from spleen and least from brain; splenic suspensions containing 1 part (dry weight) in 6,000 give complete fixation Ten minutes in a concentration centrifuge (Bauer and Pickels) at 20,000 r.p m and the supernatant fluid is filtered through a Seitz pad Such preparations of spleen are not anticomplementary after storage, the contained antigen is little affected by storage at 3° C. for 6 months, by heating at 56° C. for ½ hour, by variations in pH range from 4.5 to 9, or by dialysis." Antigens freshly prepared from guinea pig spleens react nonspecifically with a number of human sera This nonspecific factor is lost after storage for several weeks. For the past five years Smadel (personal communication[32b]) has concentrated and partially purified the splenic antigen in the following manner "After highspeed centrifugation and Seitz filtration the solution is brought to pH 4 8–5 0 with N/10 HCl and the resultant flocculant precipitate removed by horizontal centrifugation and discarded The solution is promptly readjusted to pH 7.0 and placed in a viscose tube in front of a fan at room temperature until concentrated by perevaporation to one-fourth the original volume Such preparations generally have titers of 1/128 Sufficient antigen to last for several years is usually prepared at one time and stored in 5–10 cc amounts at –20° C. or in the lyophilized state Portions of this stock which are employed for current testing may be stored at 5° C. for several months "

3 *Preparation of control hyperimmune sera* Casals[45] recommends the following method of preparing mouse sera

1) Animals are immunized with homologous tissue in order to prevent the formation of organ-and species-specific antibodies 2) the nonspecific tissue reaction is eliminated by a) centrifuging antigens at sufficiently high speed so as to sediment all or most of the tissue component (Havens *et al*[40]) or b) inactivating the sera at 60 to 65° C, according to species, thus destroying the thermolabile serum substance while hardly affecting the specific virus antibody[31]

The immunization of animals can be carried out by means of 2 intraabdominal injections of a 10 per cent formolized brain suspension, followed within 10 days by an intraabdominal injection of active virus in dilution of 10^{-1}, repeated every 4 or 5 days Within 4 to 6 weeks most animals develop a high-titer serum, which can be maintained by subsequent injections

sera and also lowered the infectious titer. More recently certain modifications of their methods have been developed by Espana and Hammon [43b, 48b] These simplify the procedure, shorten by several days the time required for preparation of a benzene-extracted antigen and result in an antigen which has a very high titer and greater sensitivity. When used in optimal dilutions they are more specific than the unextracted antigens, even though more sensitive. The method of preparation described by Espana and Hammon [43b] is as follows.

Mice are inoculated intracerebrally with a 10^{-2} or 10^{-3} dilution of virus. The mice, when moribund, are anaesthetized, bled to death and the brains removed The brains are then ground in a Waring blendor in pyrogen-free, fractionally distilled water to make a 20% suspension After storage for 3 to 4 hours at 5° C, 25 ml amounts are rapidly shell frozen in 250 ml Pyrex bottles and then lyophilized The dried tissue is then extracted with benzene for 1 hour at room temperature by adding a volume of benzene equivalent to twice the original aqueous suspension The benzene is rapidly removed by filtration through a Gooch crucible filter under high vacuum Two further benzene extractions are performed in the same filter, allowing 30 minutes at room temperature each time before applying vacuum The extracted tissue is then transferred under a hood by inverting the Gooch filter over a wide mouth stemless funnel placed in the neck of a 250 ml Pyrex bottle and then tapping the crucible filter gently till the dried powder falls free The remaining solvent is removed by applying negative pressure to the bottle Saline is next added in the amount of the original volume and rehydration permitted to occur overnight at 5° C, then after centrifugation for 30 minutes at 10,000 r.p m the supernatant is removed and Merthiolate is added to a final dilution of 1 10,000 The antigen is ready for immediate use, but for prolonged storage or shipment it is again lyophilized The liquid antigen, prior to the final lyophilization has remained unchanged in all its characteristics when stored at 5° C for at least 6 months. The final, dry product goes into complete solution rapidly and requires no centrifugation before use Its titer is the same as before lyophilization

Antigens prepared thus usually titer at 32 to 64 units With *hyperimmune* sera, highest serum titers are obtained, using the antigen in a 1:16 to 1:32 dilution (2 or 4 units) but for *human* sera of lower titer the optimal range appears to be from 1:4 to 1.8 (8 or 16 units) of antigen Greater excess of antigen tends to inhibit the reaction. As demonstrated by De Boer and Cox,[43a] these extracted antigens do not give a nonspecific reaction with Wassermann positive sera which have been inactivated at only 60° C.

Antigens are not inactivated in this laboratory for, in most instances, the titer, if reduced, will result in a loss of sensitivity of the test.[43b, 49] The benzene-extracted antigens are practically noninfectious

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Guinea pig sera are used in this laboratory.[50] Formalinized homologous brain tissue is used as inoculum for the equine viruses. For the others, two intracerebral inoculations of hamster brain virus result in high titer sera. These sera must be kept frozen; otherwise the titer falls rather rapidly.

4. *Technic of test.* Again, quoting from Casals:

Titration of Complement. Accurate titration of complement is an important step. It should be done in the presence of each antigen under the same conditions as prevail for the test proper. By our method a preliminary titration is made in saline solution, and 2 units are used in the test. Another titration is set up at the same time with antigen and with saline solution as control, which is incubated along with the test proper. Satisfactory antigens are not anticomplementary, indeed, they may enhance the titer of complement by as much as ¼ unit. It is, however, not advisable to use less than 2 units of complement (as determined in the preliminary titration in saline), since false-positive reactions may result. Complement can be kept at -76° C with little loss in titer.

The Test. Inactivated sera are diluted 2-fold beginning with 1:2 dilution, and 0 25 ml is placed in a series of test-tubes (100 × 10 mm). A series of serum dilutions is prepared for each antigen and usually 4 are used Then 0.25 ml. of antigen is added—either undiluted or at the proper dilution to be described—plus 2 units of complement in 0 5 ml The mixture of serum, antigen and complement is incubated. Following the method of Bedson and Bland,[*] the tubes are incubated at 2–4 C for 18 hours, although others prefer 37 C for 1 or 2 hours. Long incubation at low temperatures increases sensitivity without affecting specificity. Following incubation, the hemolytic system is added, which consists of 3 units of rabbit antisheep hemolysin in 0.25 ml plus 0.25 ml. of a 3 per cent suspension of packed sheep erythrocytes. Actually equal volumes of hemolysin and blood cells suspension are mixed 20 minutes before adding 0 5 ml. of the mixture to each tube The tubes are now incubated at 37 C for ½ hour and read.

Human Serum They (sera) should be inactivated at 60 C for 20 minutes; if they give a non-specific reaction, heating at 65 C should be tried; if this procedure does not eliminate a false reaction, they are then unsuitable Sera should be tested simultaneously against several antigens and, in addition, for anticomplementary effect. For specimens originating in this country the antigens of Western and Eastern equine and St Louis encephalitis and lymphocytic choriomeningitis viruses are recommended. It is advisable to begin the dilutions of human sera at 1 2 Even at this dilution specific partial fixation is of little significance unless obtained on repetition.

5. *Interpretation* Until a great deal of experience has been gained in performing the complement fixation test, it is recommended that all sera be tested twice or until two similar results are obtained, each test made on a different day, and preferably with a different antigen. Thus, certain difficulties in interpretation will obviously be avoided. Fixation at a 1:2 level must be regarded at any time with considerable skepti-

cism. Negatives, at least with the Western equine virus, must not be given the same final evaluation as a similar finding with the neutralization test.[8] If sera have been heated at 65° C. or twice at 60° C. to free them from anticomplementary or nonspecific substances, negatives, for St. Louis at least, lose much significance. No absolute time limits or quantitative levels for significance can be set any more than for the neutralization test. Two or more sera (with rare exceptions) are essential for a diagnosis, and the time and degree of rise should be in rough accordance with that shown in Table 6 It is highly advisable to perform both types of test, if possible, but it is essential to do so in all instances where the results of one are not completely beyond question.

In most sera taken 30 or more days after the onset, titers (original serum dilutions) of 1.8 to 1:64 will be obtained, but occasionally the response will be greater or less In the St. Louis-Japanese B complex (*in man*), more overlapping can be expected than with the neutralization test, especially in late convalescent sera.[8] This is particularly conspicuous in St Louis sera The overlapping is frequently exaggerated by the fact that Japanese B antigens are usually of a higher titer than those of the St. Louis virus There is also considerable overlapping with the West Nile virus It becomes obvious, therefore, that if members of this group are to be differentiated, all three antigens must be used, and final recourse to the neutralization test might be of further confirmatory value.

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RABIES

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It is during the early stages of the disease that animals are particularly dangerous; they may appear alert, friendly, and even unusually affectionate but will bite at the slightest provocation It is likewise the wild animal that suddenly loses its timidity of man that must be suspected of rabies.

The development of diagnostic methods for determining whether animals are infected with rabies was necessitated by the frequency with which human beings are bitten by animals, especially the dog As the majority of biting dogs do not have rabies, even in areas where the disease is present, it was essential to work out methods for ascertaining whether a biting dog is rabid. Although the principal emphasis is placed on examination of brain specimens from the biting animal as an aid in advising whether or not vaccination should be recommended for the person who has been bitten, it is also necessary to examine specimens from all animals suspected of having the disease in order that steps may be taken to limit the spread of rabies by quarantine or destruction of exposed dogs and cats and in order to have epidemiologic information as to the extent of the disease.

The definitive microscopic diagnosis of rabies is based on the finding of intracytoplasmic inclusion bodies, known as Negri bodies, either in the nerve cells of the suspected animal or in those of laboratory animals inoculated with brain material from this animal The nearest approach to an absolute diagnosis is the reproduction of the disease in laboratory animals by the intracerebral inoculation of brain material of the suspected animal Diagnosis by this method is too time-consuming to influence the decision regarding vaccination of persons badly bitten Hence, animal inoculation serves principally as a confirmatory test in doubtful or controversial cases where the clinical history of the offending or suspected animal is not consistent with the negative microscopic findings.

B Special Characteristics of the Virus

1 *Size* The particle size of rabies virus has been estimated at 100 to 150 $m\mu$.[1] As is the case with other of the larger viruses, rabies virus is not readily filtrable It will pass through diatomaceous earth and unglazed porcelain filters which withhold common varieties of bacteria but not through Seitz E K filter pads

2. *Resistance to physical and chemical influences* Rabies virus is ordinarily *stored* in neutral glycerol in the infected tissue in which it

G. Interpretation of Observations
H Reporting of Results

III SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS OF THE DISEASE
A. The Neutralization Test
B The Cross-Protection Test
C. The Complement Fixation Test

IV. REFERENCES

I. INTRODUCTION

RABIES is an acute specific infection of the central nervous system caused by a filtrable virus It is, as a rule, propagated in dogs and related wild animals. Man and all warm-blooded animals are susceptible. The virus is often present in the saliva of rabid animals and consequently is most commonly transmitted by a bite. Under favorable conditions the virus, when introduced into a wound, becomes established in nerve tissue and migrates to the brain where, after an incubation period of 10 days to several months, it produces an acute, highly fatal encephalitis.

A GENERAL FEATURES OF RABIES

The first symptom is likely to be some sensory disturbance related to the site of exposure, such as burning, itching, tingling, or pain Constitutional symptoms such as headache, malaise, and low-grade fever usher in the acute phase of the disease, which usually follows one of the patterns exhibited by rabid animals, that is, some patients have a prolonged period of excitation whereas in others depressive or paralytic symptoms are predominant from the beginning In the former the symptomatology is characterized by constant activity, volubility, emotional instability, apprehension, irritability, and insomnia, which may in some instances progress to a manic state In this type of the disease there is increased reflex activity of the musculature, which is responsible for the classic hydrophobic symptom, where attempts to swallow precipitate painful spasmodic contractions of the muscles of the throat As the disease progresses such paroxysms become more frequent and are likely to initiate generalized convulsive seizures Hysteria and tetanus must be considered in the differential diagnosis. In cases where the paralytic symptoms are predominant it is difficult if not impossible to make a clinical diagnosis of rabies, since the disease picture may be similar to that of poliomyelitis, paralysis following vaccine treatment, Landry's paralysis, or various types of encephalitis or myelitis associated with paralysis The disease commonly runs a rapid course, with a fatal outcome within 5 days of the onset. There is no proved instance of recovery from rabies in man One might, therefore, assume that it is always fatal When one considers the fact, however, that isolation of the virus depends on animal inoculation tests with saliva, which are rarely done even in suspected cases of rabies, it may well be that nonfatal infections do occur

only known host that can act as a carrier of rabies over an extended period without exhibiting evident illness[2] The submaxillary salivary glands are the best source of virus from tissue other than that of the nervous system. The parotid glands are less likely to contain the virus. The ability of the virus to invade the salivary glands varies with different strains, and there appears to be some difference in the ability of the virus to multiply in the salivary glands of different hosts. Rabies virus fixed for rabbits by prolonged intracerebral passage has lost, almost completely, its ability to multiply in tissues other than that of the central nervous system.

In a joint study carried on by the Georgia State Department of Health and the rabies laboratory of the International Health Division of the Rockefeller Foundation, an attempt was made to determine the frequency with which the salivary glands are invaded by rabies virus in animals naturally infected with the disease Of 28 dogs proved to be rabid by isolation of the virus from the brain, 21 (75 per cent) had demonstrable virus in the salivary glands Of 150 foxes proved to have rabies, 130 (87 per cent) were found to have rabies virus in the salivary glands In a series of 34 head of cattle studied in the same way, the virus was isolated from the salivary glands of 16 or approximately 50 per cent Strains of rabies virus isolated from foxes during an epizootic in this species[3] varied in their ability to invade the salivary glands of mice infected by intramuscular inoculation When 25 or more mice were infected with *street* rabies virus in this manner, some strains were able to invade the salivary glands in only 30 per cent of the infected mice and others would do so in 80 per cent of such mice The tropism of the virus for the salivary glands, as determined by this method, is markedly reduced by a few serial intracerebral passages of the virus in mice Rabies virus maintained in vampire bats may be especially adapted for *multiplication in the salivary glands, since these bats may transmit the disease by bite for* a few weeks or months and then become noninfectious without at any time showing evident illness[2]

Rabies virus has not been demonstrated in the blood, spleen, liver, lymph nodes, bone marrow, or sex glands of naturally infected dogs It may be present in the lacrimal glands, pancreas, and adrenal glands There is little likelihood that it will be present in the spinal fluid of human rabies patients It may be found in the salivary and lacrimal glands of these patients and in various parts of the nervous system, including ganglia, nerve trunks, spinal cord, and brain[4]

4 *Tissue changes.* Except for the occurrence of Negri bodies, the tissue changes produced by the virus are not characteristic enough to allow a pathologic diagnosis of rabies inasmuch as the lesions are similar to those found in other types of virus encephalitis.

5 *Susceptibility of species.* All of the usual laboratory animals are susceptible to rabies Infection does not appear to take place

occurs The virus will remain active for several weeks at room temperature or for months at 4° C. when preserved in this manner. As this medium inactivates or suppresses bacterial organisms, it is an excellent one for preserving infected brain material for shipping or temporary storage. Normal saline and distilled water are both satisfactory for preparing 10 per cent suspensions of infected brain for immediate testing Test virus for titration, neutralization tests and test inoculation of vaccinated and control animals should be prepared in normal saline containing 2 to 5 per cent inactivated guinea pig serum. The addition of serum to the diluent is also advised for preparation of standard virus material to be stored in the frozen state.

The resistance of the virus to physical influences, such as heat and cold, depends on the source of the infected material and the method of its preparation If infected brain tissue is suspended in normal saline or distilled water, the virus is inactivated by exposure to a temperature of 56° C. for one hour. The same material when properly desiccated may be exposed to the same temperature for an equal period of time with little loss in infectivity Though rapid freezing and storage at subfreezing temperatures may preserve the infectivity of the virus for years, repeated freezing and thawing and exposure to variable subfreezing temperatures will result in loss of infectivity Specimens stored in dry-ice cabinets must be sealed, as saturation with carbon dioxide will inactivate the virus If a standard virus preparation is to be used, the test virus should be tubed in desired amounts in pyrex glass ampules, sealed, frozen rapidly in a bath of dry ice and alcohol and stored in a dry-ice cabinet The racks used for storage of virus specimens should be so made that an ampule can be removed quickly in order to prevent deterioration of other specimens In order to obtain uniformity in tests in which frozen specimens of virus are used, the material must be thawed rapidly in a 38° C. water bath. Lyophilized specimens of rabies virus will remain infective for several years when stored at 4° C. Rabies virus is more resistant to chemical disinfectants than are bacterial emulsions. The virus is particularly resistant to phenol

3. *Distribution of virus in animals.* The virus is demonstrable in the central nervous system of nearly all animals dying of rabies When the disease is of long duration, autosterilization may take place The medulla and thalamus usually contain the greatest concentration of virus, but the cerebral cortex is also a good source of virus. The transmission of rabies in nature depends on the ability of the virus to reach and to multiply in the salivary glands of a rabid animal. The virus then is excreted in the saliva In the natural canine disease it is necessary for the animal to become vicious and bite in order to reproduce the disease. The vampire bat, *Desmodus rotundus murinus-Wagner,* is the

finding Negri bodies in the brain increases the longer the animal lives If the dog shows symptoms of rabies for a period of 5 days, however, some prefer to sacrifice the animal at that time and examine the brain for Negri bodies When virus studies are contemplated it is necessary to take into account the possibility of autosterilization of the infected tissue if the animal survives for a longer period The uniform fatality rate for rabies as compared with other diseases that might simulate it in dogs makes death a factor in diagnosis and has given rise to the practice of waiting for the animal to die This does not contraindicate the killing of an animal *in extremis* in order to obviate decomposition of the brain tissue, which might occur overnight Clinically, rabies in dogs must be differentiated from canine distemper, from fright disease due to vitamin deficiency, sometimes called running fits, and from pseudorabies due to infection with the virus of infectious bulbar paralysis, also called Aujeszky's disease There appear to be other unidentified paralytic diseases of dogs, and poisoning, either intentional or accidental, must be considered

Quarantine regulations for biting dogs usually specify 10 to 14 days' observation. The Georgia State Department of Public Health requires only 7 days' observation, and this should be sufficient, for if the animal does not show symptoms of rabies during this period it is safe to assume that the bitten person was not exposed to rabies Wild animals such as foxes, coyotes, and skunks that invade human habitation during daytime and attack and bite without provocation must be considered rabid.

Those engaged in handling rabid animals or removing brain specimens should be immunized with a full course of rabies vaccine and should be given additional injections each year unless the blood serum is found to contain a significant amount of virus-neutralizing substance. Because of the danger of serious allergic reactions, such repeat courses should be limited to 3 or 4 injections of vaccine

A. SOURCES OF MATERIAL FOR TESTING

1 *Animal specimens.* The specimen submitted for the diagnosis of rabies is ordinarily the head of the suspected animal, usually a dog It should reach the laboratory in as fresh condition as possible If the laboratory is nearby and can be reached in a few hours, refrigeration of the specimen is not necessary If the laboratory is more distant, the head of the animal should be removed at the neck and placed in a watertight container This container, in turn, must be placed in another larger one of the same type of sufficient size to permit adequate packing with ice and sawdust Metal containers, 6 to 10 inches high, 4 to 5 inches wide, and 2 to 3 inches thick, with screw-top caps, such

through the intact skin or by ingestion, but intranasal inoculation of infected saliva may produce the disease. It is difficult to infect animals by intraperitoneal inoculation, but injection of the virus into the skin, muscle, or nervous tissue, in that order, is increasingly effective in producing the disease. With rare exceptions, intracerebral injection of the virus is fatal to animals. Young birds are generally susceptible to intracerebral exposure to rabies, but mature birds are often refractory. The virus may be cultivated in tissue culture media [5] and in chick embryos,[6a] but isolation of the virus by such means is not practical as a method of diagnosis.

A close antigenic relationship has been shown to exist between rabies virus obtained from Europe, the Orient, and various sections of North, Central, and South America. All strains, including those isolated from vampire bats, show cross complement fixation, neutralization, and protection.

II. METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

The diagnosis of rabies in man and animals is based on the consideration of as many of the following factors as are available: (1) history of exposure, (2) clinical symptoms and course, (3) the termination, (4) the microscopic demonstration of Negri bodies, and (5) animal inoculation.

The history of exposure and the clinical symptoms and termination often play an important part in diagnosis, but these are mainly presumptive and attain their greatest value in lieu of positive laboratory findings. The prevention of rabies in man depends on local treatment of wounds produced by rabid animals and on postexposure vaccination. To ascertain the probability of exposure, observation of the biting animal is of utmost importance. The vaccine treatment is ordinarily recommended for persons known or suspected to have been bitten by an animal or scratched by its claws, if (1) the animal is apprehended and presents clinical signs of rabies, (2) the animal is killed and the brain is found positive for rabies by the demonstration of Negri bodies, (3) the animal is killed and, though the brain proves negative for Negri bodies, the animal is suspected of being rabid, and (4) the biting animal is not identified and the exposure occurred in an area where rabies is known to be present among animals.

It must be emphasized that when a dog or cat is apprehended after biting someone and rabies is suspected, the animal should not be killed but should be confined under the supervision of a veterinarian. This is important, because it will allow observation for clinical symptoms of rabies. If the animal is infected with this disease it will ordinarily succumb within 5 days. The probability of

3. *Saliva specimens.* Specimens of saliva[6b] are to be taken from under the tongue where the ducts of the submaxillary glands open into the mouth. The saliva may be placed in a bottle of the type recommended for brain specimens. The specimen must be kept cool, and this is done conveniently by shipment in a thermos bottle containing ice.

4. *Specimens from suspected cases of rabies in man* The laboratory diagnosis of rabies in a human patient depends on obtaining postmortem specimens of brain for microscopic examination and animal inoculation. When this is possible the methods of diagnosis are the same as for animal specimens. It may be impossible to obtain permission for postmortem examination, however, and then the isolation of the virus depends on testing the saliva, which is the only other likely source of virus Specimens of *saliva* ought to be taken as soon as the disease is suspected, because there is likely to be a decreased production of saliva as the infection progresses. Though a negative result from animal inoculation with saliva does not rule out rabies, a positive result does prove the diagnosis in the absence of postmortem examination. Unless frequent attempts are made to isolate the virus from the saliva, there is little chance of proving that the disease is not always fatal Where known exposure to rabies has occurred and where any ill-defined symptoms, such as headache, low-grade fever, malaise, or local reaction about the site of exposure are noted within the first few weeks after the bite, saliva specimens may be taken for animal inoculation in order to determine whether abortive infections do occur

The *spinal fluid* cell count is ordinarily within normal limits in human rabies, and this may be useful information in arriving at a clinical diagnosis On the basis of animal experimentation, if the cell count is elevated it may be possible to isolate the virus from the spinal fluid Under such conditions centrifugation of the spinal fluid will show that the virus is to be found in the cellular sediment but not in the supernate. It is extremely unlikely, however, that the virus will be present in the spinal fluid in human rabies[4]

Though acute and convalescent phase *blood specimens* are of great value in the diagnosis of some virus infections, they are of little or no value for the diagnosis of rabies The taking of acute phase blood specimens should nevertheless be routine in cases of suspected rabies. In the event that the patient recovers, the specimens will provide a means of ruling out other diseases In cases where the vaccine treatment was not given it may be possible to demonstrate a rise in titer of

as those used commercially for marketing varnish and metal polish, may be filled with ice and packed about the specimen container to prevent the wetting of the packing material.

The package containing the specimen to be examined should be addressed to the nearest laboratory of the state health department. It should be labeled, "Rabies Suspect, Rush," and the type of animal head should be specified. Shipment should always be made by express. The United States postal regulations prohibit the conveyance of such material by mail. The Railway Express Agency, which is one of the largest express companies in the United States, issues the following rules and regulations governing the shipment of heads of dogs or other animals by express to diagnostic laboratories:

a. Agents must not accept for transportation the head of a dog, or any other animal, sent to state boards of health for rabies examination, unless it shall have been prepared for shipment as hereinafter provided

b The head of a dog or other animal so shipped must be placed in a tin can or other metal container, which will not permit the leakage of fluids, such container shall then be placed in a second metal container with ice packed around it; such outside container must be so constructed that it will not permit the leakage of the ice water.

c. All such packages must be labeled CAUTION—This package contains the head of a dog (or name of other animal) suspected of having died of hydrophobia

d. Such shipments tendered on Saturday, which cannot reach destination early enough for delivery on that day, and would, therefore, remain in the express office over Sunday, must be refused, and shipper requested to pack in ice and hold until Monday, so that they can be delivered without delay at destination

e Require prepayment of charges on shipments of this kind

2. *Brain specimens for animal inoculation* When microscopic examination of the brain of an animal suspected of having had rabies fails to show the presence of Negri bodies and inoculation of test animals with suspensions of the brain cannot be performed locally, specimens may be sent by mail to a laboratory of the state health department for infectivity studies. A sturdy glass vial of the vaccine bottle type, equipped with a sleeve-type rubber stopper, should be used for such specimens Small portions of the medulla, thalamus, and cerebral cortex should be removed from the brain specimen with reasonable aseptic precautions Undiluted neutral glycerol is the best preservative The vial containing the specimen must be packed in absorbent cotton and prepared for shipment like ordinary bacteriologic specimens in double metal containers so constructed that the specimen bottle will not be crushed.

b Grasping the muzzle with one hand, make a midline incision and cut away the skin and muscle so as to expose the skull to the base of the ear on each side

c With the meat cleaver, using short chopping blows, cut through the skull, at first transversely just behind the eyes, then laterally on each side as far back as the base of the ear

d With the point of the cleaver, or with the lion-jawed forceps, pry the calvarium up and back, exposing the brain

e Split the meninges in the midline and push them aside

f Holding the muzzle of the head upright, insert a knife blade under the frontal lobes and work downward, severing the cranial nerves to the brain stem At this point the brain tends to fall out of its own weight

g The entire brain, including at least parts of the medulla and cerebellum, is thus shelled out and delivered into a suitable container The ordinary paper picnic plate about 8 inches in diameter with fluted rim is ideal for this purpose Such plates are cheap and may be destroyed with the brain when the examination is completed.

h Wrap the head in paper, drop it in a garbage can; and immerse all instruments in the jar of disinfectant

i Finally, remove the gloves and carry the brain on the paper plate into the examining room

j If two or more heads are received at the same time, open one and completely dispose of it with proper labeling before the second is unpacked Otherwise, there is danger of an embarrassing error

k If animal inoculations are to be included in the diagnostic study, all instruments must be boiled between each operation, as phenol solution cannot be depended upon to destroy rabies virus on the instruments during a limited period of exposure

l In order to reduce bacterial contamination and to destroy ectoparasites, it is advisable to immerse animal heads in 1 to 5 per cent lysol solution before opening the calvarium

m Discarded animal heads and brain material should be incinerated This may be done with proper equipment in the laboratory or by arrangement with the sanitary department of the city.

In spite of instructions cautioning against mutilating the head, every laboratory at times receives specimens badly lacerated by gunshot or crushing blows Damaged brains may prove satisfactory for examination if the contents of the brain cavity are removed and placed on the paper container as usual The container is then placed in the sink, and the blood clots and debris are washed away, using a chemical water bottle filled with normal salt solution The portions of brain tissue which remain intact are then placed on a clean container

2 *Source of material for microscopic examination.* While Negri bodies may be present in any portion of the brain containing nerve cells, they are, as a rule, most numerous and characteristic in the

neutralizing substance for rabies virus in the course of the illness Specific complement-fixing antibodies can be demonstrated in the blood of experimental animals immunized with rabies virus, beginning about the 10th day after the first dose of vaccine and reaching a maximum titer in 3 to 4 weeks. At the end of 2 to 3 months, the complement-fixing antibodies will have disappeared, but virus-neutralizing substance can be demonstrated in high titer. The complement fixation test has not been shown to be applicable to the diagnosis of rabies in man or animals.

B. Preparation of Materials for Inoculation or Examination

1. *Animal heads.* Laboratories that have to handle large numbers of animal heads should provide a small isolated room for removing brains. A floor space of 50 square feet is adequate. The room should be well lighted and equipped with floor drain and forced ventilation. A laboratory assistant can be taught to remove brains.

Equipment

a. Knives Stout butcher knives of medium size (6- to 8-inch blades) are excellent A small dissecting knife is also useful.

b Butcher's cleaver If kept sharp, this is better than a saw for opening the calvarium. The cleaver is also easier to keep clean and sharp.

c. Meat cutter block. In laboratories that handle large numbers of heads, this is important Improvised blocks may be quite as satisfactory

d. Disinfectant jar. This should be filled with 5 per cent lysol or suitable noncorrosive disinfectant solution in which the instruments may be kept between operations

e. Forceps. Lion-jawed, bone-holding forceps *

f. Heavy rubber gloves Of the type known as acid handlers' gloves †

g Goggles These may be worn as an added precaution to protect the eyes against flying bits of brain and tissue

h Sink This should be equipped with drain boards and hot and cold water

i. *Garbage cans*

j Paper plates for receiving the brains These are very useful if many heads are handled, for reasons given below

Procedure

a After donning the heavy gloves, unpack the animal's head and place it on the block

* Such forceps may be obtained from Kny-Scheerer Corporation, 21-09 Borden Avenue, Long Island City, N Y

† An excellent glove for this purpose is manufactured by United States Rubber Company, Latex Industrial Gloves, U S No 1307

Stain formula

Basic fuchsin,* saturated absolute methyl alcohol solution ......2–4 ml
Methylene blue, saturated absolute methyl alcohol solution .... 15 ml.
Methyl alcohol (absolute acetone-free) . .. .25 ml

Because of some variability in the dye content of different lots of stain, it is preferable to use an excess of the powdered dye so as to leave a definite undissolved residue, for example, 20 gm of basic fuchsin or 10 gm of methylene blue to 500 ml of absolute, acetone-free, methyl alcohol Only methylene blue of the Med USP type is satisfactory, and the BX grade has proved to be an excellent stain for the demonstration of the inner structure of Negri bodies

The saturated methylene blue solution and the methyl alcohol are mixed first, and 2 ml of the saturated basic fuchsin solution is added. A trial stain is made Macroscopically, the properly stained smear when held up to the light should appear reddish-violet in the thinner areas, shading into purplish-blue in the thicker portions If in the trial stain the thinner parts are bluish, 0 5 ml more fuchsin solution is nearly always sufficient The mixed stain seems to be improved after standing 24 hours, and thereafter keeps indefinitely if protected from evaporation, which tends to make the fuchsin become too dominant. The addition of absolute methyl alcohol to the evaporated stain will usually restore the proper balance to the two dyes

1. *Procedure for staining*

a. Prepare smears or impressions in the usual way No preliminary fixation is required

b. While the preparation is still moist, immerse it in the staining solution and remove at once

c Rinse in tap water, dry without blotting, and examine.

In some regions tap water is not satisfactory for use in rinsing the stained preparation. Whether or not it is suitable can be determined by comparing preparations rinsed with tap water and others rinsed with triple-distilled water containing M/150 phosphate buffer, pH 7.0 The entire process of staining can be completed in a few seconds The stained slide may be dried by warming and waving through the air. The low power (16 mm) objective should be used first in examining the stained smear Thin areas showing numerous large nerve cells well spread out can be thus located and then examined with the oil immersion lens The Negri bodies are stained cherry red and stand out in sharp relief The basophilic or blue-staining inner structure of the Negri bodies is well stained The cytoplasm of the nerve cells stains blue to purplish-

* Basic fuchsin obtained from Coleman and Bell (dye content at least 92 per cent) has given good results

Ammon's horn or hippocampus. Therefore, this structure should be examined first

Place the brain base downward and make a deep longitudinal incision with scissors or knife into one hemisphere just lateral and parallel to the superior longitudinal fissure. Cut deep enough to expose the lateral ventricle The hippocampus can be recognized as a glistening white, cylindrical body bulging up from the ventricle floor. The Ammon's horn may be further identified by sectioning it transversely, revealing its characteristic concentric light and dark zones.

C. Preparation of Smears and Impressions

Smears

a. With small scissors, cut through the Ammon's horn transversely. The cut surface will show concentric light and dark zones.

b. With the same scissors, clip from the cut surface a small segment about a millimeter thick Transfer this to a clean slide near one end.

c. Superimpose a second slide on this, and flatten the tissue out to a thin layer by pressure.

d. Draw the top slide lengthwise over the other, leaving a thin elongated smear on the bottom slide. The smear is now ready to be stained

Impressions

a. Remove a 1-mm.-thick cross section of the Ammon's horn with scissors or scalpel

b. Place the section flat upon the porous dry surface of a wooden tongue depressor or paper toweling and press it down gently at the edges so as to obtain firm adhesion.

c. [illegible] it lightly against [illegible] . [illegible]is should leave a [illegible] he section from the absorbent surface

d. Make several impressions on the same slide. Use fresh cross sections for a second slide if desired

e. Stain the impression

D. Staining of Smears and Impressions

The staining solutions most commonly employed are modifications of van Gieson's stain, which was originally an aqueous mixture of methylene blue and rose aniline violet[7] Later, basic fuchsin was substituted for rose aniline violet by Frothingham[8] and by Williams.[9] The modification now in general use is that of Sellers[10] By this method preliminary fixation is not necessary, since the dyes are dissolved in methyl alcohol instead of water so that the staining solution also acts as a fixative.

Stain formula

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Methylene blue, saturated absolute methyl alcohol solution . . . . 15 ml.
Methyl alcohol (absolute acetone-free) . . . . .25 ml

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* Basic fuchsin obtained from Coleman and Bell (dye content at least 92 per cent) has given good results

blue, depending on the balance of the fuchsin and methylene blue; the nuclei and the nucleoli of the nerve cells stain a deeper blue; the stroma is stained rose pink; nerve fibers stain a deeper pink, the neural sheaths do not stain; bacteria, if present, are stained intense blue; and erythrocytes copper color.

2. *Distribution of Negri bodies.* While Negri bodies are as a rule more abundant and more characteristic in the Ammon's horn than elsewhere, they may often be found in the gray matter of the cortex or in any area of the cerebellum, medulla, thalamus or cord where nerve cells are present. They are usually abundant and of large size in the Purkinje cells of the cerebellum. *Their distribution is often irregular.* They may be absent or scarce in the Ammon's horn and yet be found in the cortex, particularly in the region of the sylvian fissure, the cerebellum and the cranial nerve nuclei of the medulla. Therefore, *no specimen should be reported as showing no Negri bodies until preparations* from the Ammon's horn and cortex of both hemispheres and from the cerebellum are thoroughly examined No fewer than 50 nerve cells in each preparation should be studied. Thin portions of the film showing nerve cells well spread out are to be selected for examination.

E PREPARATION AND STAINING OF PARAFFIN SECTIONS

Because of the longer time required for their preparation, sections are rarely employed for the diagnostic demonstration of Negri bodies. Smears and impressions require at most only a few minutes, although the shortest reliable sectioning procedure so far devised requires several hours and necessitates the service of a technician trained in pathology. If Negri bodies cannot be found in smear or impression preparations there is little chance of making a microscopic diagnosis by any method. Hence sections are employed chiefly for confirmatory studies of general pathology in doubtful cases and for research purposes

For the demonstration of Negri bodies in paraffin sections, the brain tissue should be fixed in Zenker's fluid To the desired amount of fixative add glacial acetic acid to a concentration of 5 per cent. The blocks of tissue must not be more than 2 to 3 mm. thick in order to obtain good fixation. Following fixation for at least 4 and preferably 24 hours, the tissue is washed thoroughly in running water and may then be stored in 80 per cent alcohol After embedding in paraffin the sections must be treated with Lugol's solution and sodium thiosulfate to remove the corrosive sublimate crystals deposited in the tissue by the

fixative. The phloxine and methylene blue stain and the Wolbach modification of Giemsa's stain, as given by Mallory [11] are suitable for the demonstration of Negri bodies in paraffin sections. Giemsa stain may now be prepared using a known amount of the azure dyes.[12]

F. Animal Inoculation

Methods for the isolation and identification of rabies virus have been known for a long time. Until a few years ago the guinea pig and rabbit were considered the most suitable animals for infectivity studies. Recent studies have shown that the white laboratory mouse is the best experimental animal for the isolation and identification of rabies virus [13] The advantages of the mouse are its low cost, making possible the use of several animals for the testing of one specimen; the relatively short incubation period of rabies in this animal; and the consistency of the production of Negri bodies in the brains of mice inoculated intracerebrally with *street* rabies virus. Any of the various strains of white mice are equally suitable as test animals.[14]

1. *Materials and methods.*

a The specimen ordinarily used for test inoculation consists of *brain tissue.* It is usually possible to keep such specimens under refrigeration until they are prepared for animal inoculation. Fresh or glycerolated specimens are prepared by grinding them in a mortar and making a 10 per cent suspension in normal saline or distilled water. An abrasive such as sterile sand or alundum may be used, but this is not necessary. After the tissue has been ground to a pulp a portion of the diluent is added; the remainder of the diluent is added only after the tissue is evenly dispersed The brain suspension is then decanted into a sterile pyrex test tube and subjected to centrifugation at approximately 2,000 r p m for 5 to 10 minutes The supernatant fluid is used for test inoculation.

(1) Mice When tests are made in *mice* the dose is 0 03 ml, given intracerebrally by means of a 0.25 ml tuberculin syringe and a 1/4 or 3/8 inch, 26- or 27-gauge needle. No antiseptic is necessary over the inoculation site, as bacterial infection develops rarely unless the material used for inoculation is contaminated. Where contamination is evident from examination of the smears or impressions from the brain specimen, the selected portions of brain tissue may be placed in pure neutral glycerol for 48 hours or suspended in normal saline containing 0.5 per cent phenol and left at refrigerator temperature for 24 hours before

testing. This treatment ordinarily reduces the activity of the bacteria to such an extent that animals inoculated intracerebrally with the material do not die of bacterial meningitis. The mice are ordinarily anesthetized by dropping them into a battery jar containing a wire mesh platform under which cotton is placed so as to allow rapid evaporation of the ether when this is poured into the jar. The head of the anesthetized mouse is held firmly against the work table by the thumb and index finger of one hand and the syringe is held with the other hand, using the index finger on the plunger and stabilizing the barrel of the syringe between the thumb and 3d and 4th finger. The forearm should rest on the table to stabilize the arm, and the needle is inserted in the center of one of the parietal areas. Mice seldom die from the injection procedure. It is best to use at least 4 mice for each specimen. Mice show no appreciable decrease in susceptibility to intracerebral inoculation of rabies virus with increasing age. It is preferable to use mice between 3 and 6 weeks of age, as older one are more difficult to inoculate because of the hardening of the bony structure of the calvarium.

If the specimen is positive for rabies, some of the mice usually will show tremulous muscular activity, inco-ordination, or paralysis between the 6th and 8th day after inoculation Other symptoms include humping of the back, ruffing of the fur, and conjunctivitis Inoculated mice should be observed daily, and if they appear ill the preconvulsive tremor exhibited in the early stages of rabies may be elicited by lifting the mouse by the tail with a pair of long dressing forceps. Generalized convulsive seizures are common, and the animal may die during such a seizure. The majority of mice infected with *street* virus rabies develop flaccid paralysis of the legs; this progresses to complete prostration during which the animals show labored and irregular respiration Negri bodies can often be demonstrated in the brain on the 5th day after inoculation with *street* rabies virus After the development of symptoms, Negri bodies are usually numerous and can be found throughout the brain.

The microscopic examination of mouse brains for Negri bodies is necessary in order to establish the diagnosis. Impression preparations are made from a cross section of the brain, and with a little experience it is possible to cut across the brain so that the hippocampus will be exposed on the surface of the cross section. The preparations are then made and stained as described previously There are sometimes found

in the nerve cells of mouse brains small intracytoplasmic inclusion bodies which are evidently due to some natural disease of mice. These nonspecific inclusions have no basophilic inner structure; they are round and uniformly small, and though they stain pink with Sellers' stain their texture is smooth as compared with the finely granular appearance of Negri bodies. Their distribution is largely limited to the thalamus and hypothalamus In our experience these inclusion bodies are most commonly encountered at times when some of the stock mice exhibit natural respiratory disease.

In routine mouse inoculation tests surviving mice are ordinarily discarded and destroyed on the 30th day following intracerebral inoculation Infrequently the incubation period of rabies in mice inoculated intracerebrally with brain specimens from natural cases, will be prolonged to 2 or 3 weeks. To a certain extent, such prolongation of the incubation period must be due to a low concentration of virus There seems to be some variation, however, in the incubation period in mice inoculated with different strains of *street* rabies virus not due solely to the concentration of virus In mouse inoculation tests performed at the Georgia State Department of Health, an incubation period of 65 days was noted for one mouse injected intracerebrally with *street* rabies virus

(2) Rabbits and guinea pigs may be used for diagnostic tests for the presence of rabies virus, but this involves a more tedious technic than that required for mice since it is necessary to make a small incision in the skin of the rabbit or guinea pig and to trephine the skull before introducing the needle and injecting the test material. A shoemaker's awl or a large-sized hypodermic needle may be used to make the perforation in the calvarium The usual dose of inoculum for guinea pigs is 0 1 ml and for rabbits 0 25 ml In these animals the incubation period of *street* virus rabies is usually longer and more variable than it is in mice, and rabbits and guinea pigs used as test animals should be kept for observation for at least 60 days In most instances the guinea pigs will develop symptoms of rabies by the 10th day after exposure, but rabbits seldom show symptoms of the disease before the 12th day In general, the incubation period of *street* virus rabies is 10 to 20 days for guinea pigs and 15 to 30 days for rabbits An incubation period of more than 30 days is not uncommon in both types of animals. The disease picture in guinea pigs and rabbits is much the same as that described for mice Laboratory animals infected with

rabies virus from natural cases seldom show the furious form of the disease if the virus is given by intracerebral injection. Viciousness is rarely noted in white mice infected with the disease but guinea pigs and rabbits, especially the latter, may attack and bite and therefore it is advisable to wear heavy leather gloves when handling such animals after inoculation. Mice need not be picked up by hand, since long forceps are well suited for handling these animals.

(b) Specimens of salivary gland are prepared and tested in the same manner as brain specimens.

(c) Saliva is seldom satisfactory for intracerebral injection unless diluted, inasmuch as this material is very toxic to mice and other animals when injected into the brain Untreated saliva is likely to produce bacterial meningitis, though relatively few organisms can be demonstrated in smears. Undiluted saliva can be tested by intramuscular injection into guinea pigs and rabbits. A dose of 0 1 ml. injected into each thigh muscle should be given to at least two animals. These animals should be observed for at least 90 days before they are destroyed If mice are to be used, the saliva should be diluted 1 in 10 and 1 in 100 in normal saline containing 2 to 5 per cent inactivated guinea pig or rabbit serum, and now that penicillin is available this may be added to a concentration of 1,000 units per ml. At least 4 mice should be injected intracerebrally with each of the 2 dilutions

(d) Spinal fluid tests are probably of little value for the diagnosis of rabies. but an effort should be made to test this fluid in all cases of encephalitis whether or not rabies is suspected.

When animals injected with specimens of brain show symptoms of encephalitis and Negri bodies cannot be demonstrated and further study is indicated, the entire brain of some of the animals should be removed with sterile precautions When the test animal is a mouse, it is killed with chloroform and the body is fastened to a dissecting board with push pins. If desired, the entire mouse may be dipped in lysol solution, but the usual procedure is to wet the head and back with 70 per cent alcohol With one set of scissors and forceps the skin is reflected from the calvarium. It is convenient to grasp the skull of the mouse with the forceps in the eyesockets and then puncture through the calvarium with a small pair of curved, pointed dissecting scissors and cut around the calvarium. The brain is removed by cutting the olfactory nerves and lifting out the brain with the scissors Small petri dishes are convenient for handling the specimen. If further passage is

not desired immediately the specimen may be stored in the freezing compartment of a refrigerator or in a deep freeze unit.

It is necessary to be vigilant in order to prevent a mix-up of virus, especially in laboratories where strains of *fixed* rabies virus are maintained for vaccine production. It must be remembered that phenol or cresol solution cannot be depended upon to kill rabies virus on instruments which may be used over again for another specimen. The instruments must be boiled for 5 minutes in a small tray of water. Where sufficient instruments are available, a ready supply of sterile scissors and forceps can be kept on the work table in metal boxes with front-lift tops having overhanging edges and two partitions running from the front to the back of each box. Straight and curved scissors and forceps are placed in these compartments with the points to the back, and the box and contents are sterilized in a dry-heat oven. A temperature of 160° C. for 2 hours is recommended. Syringes and needles may be sterilized in the same oven by placing them in test tubes with cotton at the bottom and then by stoppering with cotton. When rinsing syringes and needles after use, technicians should be trained always to immerse the point of the needle in the water so that the spray will not be dispersed into the air.

2 *Other viruses to be ruled out.* Mice inoculated with brain specimens may become infected with a variety of specific viruses and bacteria which will produce encephalitis or meningitis and symptoms of tremulous activity, convulsions and paralysis Among the viruses that may be encountered are those of pseudorabies or Aujeszky's disease (cow, rat, and dog), lymphocytic choriomeningitis (man, rodent, and dog), and equine encephalomyelitis (man, horse, and mule). Among bacterial diseases, infection with the *Listerella monocytogenes* organism (cow and pig) is commonly confused with a virus disease Toxoplasmosis is another disease that may be encountered

3. *Immunologic and serologic identification of virus* Rabies virus isolated from naturally infected animals or man is ordinarily readily identified by the demonstration of Negri bodies in the brains of animals infected by intracerebral inoculation of the isolated virus. This identification may be checked by cross complement fixation, neutralization and protection tests using known immune sera and a standard strain of *fixed* rabies virus if desired Such tests are of particular value in establishing the identity of *fixed* strains of rabies virus where specific inclusion bodies do not develop

G. Interpretation of Observations

An immediate diagnosis of rabies by microscopic examination of the brain of man or animals naturally infected with rabies is not regularly obtained. Only about 30 per cent of human victims of rabies will show Negri bodies in the brain when examined post-mortem. In a series of routine specimens of dog brain received by the Georgia State Department of Health during 1937, a total of 771 was positive for rabies by mouse inoculation and, of these, 81 (10 5 per cent) proved negative for Negri bodies by immediate microscopic examination. Among 188 dogs infected with rabies by intramuscular inoculation of *street* rabies virus and allowed to die, 87 per cent of those that lived over 3 days after the onset of the disease proved positive for Negri bodies. Only 48 per cent of the dogs dying of paralytic rabies proved positive for Negri bodies[15] Biting dogs, those with furious rabies, tend to live 3 or more days after the onset of symptoms, and a positive diagnosis is usually possible in about 90 per cent of such animals by microscopic examination of the brain An absolute diagnosis is possible only by animal inoculation and by the demonstrations of Negri bodies in the brains of infected animals. In rare instances dogs dying of rabies have little or no active virus in the brain tissue as determined by animal inoculation This finding can best be explained by the fact that in experimental animals there is a gradual decrease in the amount of active virus in the brain after the first 48 hours of the disease. Among mice inoculated with brain material from rabid animals living 5 or more days there are frequently survivors, and titration of the inoculum in such instances shows a low concentration of active virus Rabid dogs may live as long as 11 days after the onset of symptoms The same is true of man The medulla is the best source of virus in such cases, and it is advisable to inject 10 mice with the specimens if it is known that the disease was of long duration. In such cases only 2 or 3 of the 10 inoculated mice may develop the disease. In rare instances it has been impossible to demonstrate any active virus, although the disease picture was characteristic and there was known to have been natural or experimental exposure. In dogs that are allowed to die, prolonged exposure to high temperature, such as is incurred by their lying in the sunlight or in very warm enclosures, may reduce the amount of active virus in a relatively short period of time

H. REPORTING THE RESULTS

From the standpoint of public health practice, the finding of typical Negri bodies in the brain specimen examined is diagnostic of rabies and needs no further confirmatory test. Written or telegraphic reports may read "The . ... head submitted by you . ... 194.... shows rabies present," or "shows positive evidence of rabies" It is customary in most state laboratories to report all positive findings by wire, whether or not this has been requested The telegraphed report is followed by a mailed report which should include such additional information and advice as may be needed for the protection of persons and livestock exposed.

When Negri bodies are not found, the report may in effect be worded thus "The .. . head submitted by you .... 194.... shows no evidence of rabies. This does not exclude the possibility of its presence The clinical history of the animal should be carefully considered Consult your health officer or your physician, and follow his advice" Many public health laboratories enclose with all negative reports a questionnaire which, if answered fully, supplies the important features of the past and present clinical history of the animal in orderly sequence, thus enabling authorities to advise more intelligently as to the need for antirabic treatment of persons bitten or exposed

If the brain is too badly mutilated or decomposed to permit satisfactory examination, the report may read as follows "The . .. . .. head submitted by you . . 194 was too badly lacerated (or decomposed) for satisfactory examination for rabies Consult your health officer or physician, and follow his advice" In such instances it is all the more important to enclose with the mailed report some form of clinical history questionnaire

The laboratory report, whether positive or negative, offers an excellent vehicle for the distribution of educational literature on the subject of rabies. The occasion is the psychological moment to drive home correct information on this subject.

III SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS

Owing to the uniform fatality of rabies in man and to the fact that the virus can be identified by the demonstration of Negri bodies in the brain of the person dying of the disease or in animals infected with the brain material, immunologic tests are of little value for the diagnosis of rabies Neutralization and complement fixation tests might be applicable to the diagnosis of rabies if it were not for the routine use of post-exposure vaccination, which, in itself results in the production of specific serum antibodies The demonstration of a rise in titer

of such specific immune substances in the blood, during the course of the disease, is possible in untreated cases of rabies. Immunologic tests are therefore largely restricted to the identification of *fixed* rabies virus, which does not produce Negri bodies, and for research purposes.

A. THE NEUTRALIZATION TEST

For this test it is preferable to have a standard virus, so that undiluted and various 10-fold dilutions of serum may be tested against a given amount of active virus. The alternative is to titer the virus, as obtained from a fresh brain specimen, in normal and immune serum for comparison with the unknown serum. Preliminary titration of one or more ampules of a standard virus stored in the frozen state makes it possible to use a certain amount, ordinarily 20 and 200 LD_{50} per 0.03 ml., which is the inoculum for mice. When this virus is mixed with an equal volume of serum the concentration will be 10 and 100 LD_{50} per 0.03 ml. The amount of virus remains constant, and the serum is tested undiluted and in 10-fold dilutions in normal saline, through 10^{-3}. The normal serum control is tested against 10 and 100 LD_{50} and the immune serum control against 100 LD_{50} in the full range of dilutions used for the unknown sera. A volume of 0.3 or 0.5 ml. of the virus dilution is added to each tube, followed by addition of an equal volume of the serum or serum dilution to be tested Incubation of this mixture for one hour at 38° C. and another hour at 4° C., with agitation of the tubes at 15-minute intervals, has proved sufficient to allow satisfactory neutralization of the virus by known immune serum. It is convenient to use short test tubes so that the syringe may be filled without decanting. It is also preferable to have a separate syringe and needle for each tube, as these can then be left standing in the tubes, and if some of the inoculated mice die shortly after the test injection, replacements may be made. At least 4 mice should be injected intracerebrally with 0.03 ml. of each serum-virus mixture. Inoculated mice may be discarded 21 days after injection.

There is no appreciable loss in virus titer during the test period, and there are rarely any survivors in the 10 LD_{50} control group Survival of more than 50 per cent of the mice in the 100 LD_{50} group tested against undiluted serum is regarded as satisfactory evidence of antibody to rabies The use of a standard virus derived from rabbits prevents the introduction or maintenance of a mouse disease agent

in the test virus material. The virus should be free of bacteria, as determined by culture.

Control normal and immune sera are ordinarily obtained from guinea pigs. To obtain a standard immune serum, guinea pigs are vaccinated with a phenolized live virus vaccine. Guinea pig brain infected with *fixed* rabies virus is homogenized in normal saline containing 1.0 per cent phenol to make a 20 per cent suspension. This suspension is left at 4° C. for 1 week and then diluted to a 10 per cent suspension by adding an equal volume of normal saline. It is then ready for use. The guinea pigs to be immunized are given 3 intraperitoneal injections of 2 ml. of this vaccine, at weekly intervals, and bled while fasting 1 week after the last dose of vaccine. The blood serum from several animals immunized in this way may be pooled and stored in appropriate amounts in the frozen state or in vaccine bottles with sleeve-type rubber stoppers so that the desired amount of serum may be removed with a syringe and needle. Merthiolate may be added to a concentration of 1:10,000 without affecting the specific reaction of the serum. Immune serum prepared in this way will ordinarily neutralize 100,000 LD_{50} of *fixed* rabies virus per 0 015 ml. It will keep for at least 2 years at refrigerator temperature without appreciable loss in its ability to neutralize rabies virus.

B The Cross-Protection Test

Five- to 6-week-old mice should be used for protection tests, since they are more readily immunizable than those 3 to 4 weeks of age. At least 12 and preferably 25 animals should be used for each vaccinated and control group A vaccine is prepared from infected mouse brain by the same method as that described for the immunization of guinea pigs A complete cross-protection test may be done by immunizing one group of mice with a known strain of rabies virus and another with the unknown virus The mice to be immunized are given 3 intraperitoneal injections of 0 25 ml of the vaccine, at weekly intervals, and 1 week after the last dose of vaccine they are challenged by intramuscular injection of the test virus The challenge virus is prepared from infected mouse brain by grinding in normal saline to make a 10 per cent suspension of brain This suspension is allowed to settle for 1 hour, and the supernate is then removed and used as the test virus. A dose of 0 03 ml of this material injected into each thigh muscle should give a uniformly high mortality rate. Centrifugation reduces the infectivity

of the supernate of a suspension of infected brain material as determined by intramuscular or intraperitoneal inoculation. Mice immunized to rabies in the manner described above should all resist peripheral inoculation with rabies virus. The challenge virus may be given by intracerebral inoculation. By this method it is necessary to titrate the virus in normal and immunized mice. Evidence of protection is obtained if there is a significant difference in the titer of the virus in immunized as compared to control mice. This method has been adapted for testing the immunizing potency of rabies vaccine.[16]

C. THE COMPLEMENT FIXATION TEST

The complement fixation test with mouse brain antigen, as described elsewhere in this book for the study of neurotropic viruses, is applicable to rabies. Blood serum from guinea pigs vaccinated in the same manner as those vaccinated for the purpose of obtaining immune serum for the neutralization test will show a good titer of complement-fixing antibodies.

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HERPES SIMPLEX

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  - 1. Herpes simplex
  - 2 Keratoconjunctivitis
  - 3 Acute infectious gingivostomatitis
  - 4 Kaposi's varicelliform eruption
  - 5 Traumatic herpes
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- B The Virus and Its Pathologic Lesions
  - 1. Size
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II Methods of Isolation and Identification of the Virus
- A Special Precautions
- B Sources of Material and Collection of Specimens
  - 1 Swabs from ulcers of mouth, eye, or genitalia
  - 2 Vesicle fluid
  - 3 Saliva
  - 4 Cerebrospinal fluid
  - 5 Brain or spinal cord
  - 6 Blood
- C Animal Inoculation
  - 1 Rabbit
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    - b Intracerebral route
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I. INTRODUCTION

SINCE 1919[1] it has been recognized that the virus of herpes simplex could be isolated from "fever blisters" or "cold sores" (herpes simplex, herpes febrilis), and the frequency of their occurrence makes it one of the commonest virus agents associated with human disease. Within the last ten years, however, it has been realized that this virus plays a much wider role in human pathology than as the causative agent of the clinical entity of herpes simplex. At present, it is known to be the causative agent of six disease entities, if all the various types of herpes simplex, such as herpes labialis and herpes progenitalis, are included under one heading

A. GENERAL STATEMENT OF THE DISEASES CAUSED BY THE VIRUS

1. *Herpes simplex*[2] is an eruption of sharply defined, closely grouped, thin-walled vesicles on an erythematous base which may occur on any part of the skin but has a particular predilection for mucocutaneous junctions and frequently recurs in the same place.

2. *Keratoconjunctivitis*[3,4] is a conjunctivitis usually accompanied by some corneal involvement The conjunctiva shows a predominance of mononuclear cells on epithelial scrapings, negative bacterial cultures, a late-appearing, if any, purulent exudate and, often, a palpable preauricular lymph node The coincidence of herpetic vesicles on the eyelids may be the clinical clue to the diagnosis Perhaps the most commonly recognized manifestation of corneal involvement is the so-called "dendritic" ulcer of the cornea, although other types of ulceration occur It is

characteristic of these ulcers to recur, but because they are superficial they rarely lead to scarring, unless secondarily infected

3 *Acute infectious gingivostomatitis*[5] is an ulcerative stomatitis and is most frequently seen in children between 1 and 3 years of age.

4. *Kaposi's varicelliform eruption*[6] One type has recently been given the name "eczema herpeticum"[7] The clinical diagnosis depends on the presence of vesicles over a portion, or portions, of skin previously afflicted with atopic eczema

5. *Traumatic herpes*[8] consists of vesicles, sometimes of the giant variety, recurring in the site of a previously traumatized area of the skin, for example, the site of a gravel graze of the palm of the hand

6 *Acute infection of the central nervous system* may take the following forms (*a*) an acute fatal encephalitis,[9] (*b*) an acute meningitis of the so-called benign aseptic or lymphocytic variety[10]

Herpes virus occupies a peculiar position among agents noxious to man in that an almost perfect mutual adaptation between the human host and this parasite has been realized A very high proportion, 70 to 90 per cent of adults,[11] show, by the presence of antibodies in their blood, that they have been infected by the virus, yet few give a history of having suffered from any of the conditions mentioned above Nevertheless, in spite of the presence of antibodies, certain people have attacks, often recurrently, of one or another of the clinical syndromes just mentioned. It has come to be realized from the investigations of the last 10 years that the reaction of the patient to the first exposure to the virus may be a violent one, giving rise to an attack of acute infectious gingivostomatitis,[11, 5] eczema herpeticum,[6, 7] or even a fatal encephalitis[9]

From some patients with clinical manifestations of primary infection with the virus, for example, acute infectious gingivostomatitis, it is possible to get a history of contact with someone suffering from herpes, usually herpes labialis, to provide a source of the virus In others, no obviously infected contact can be found. In such cases it must be assumed that one of the contacts is carrying the virus asymptomatically, probably in the saliva. It is known that virus can be isolated from the saliva of apparently normal persons, but the factors that influence the susceptibility of a recipient of such infected saliva, by droplets or otherwise, are imperfectly understood. Certainly upper respiratory infection and trauma are commonly associated with the onset of an acute gingivostomatitis, whereas trauma of the skin in the form of eczema or mechanical abrasions paves the way for a superadded herpetic infection (Kaposi's varicelliform eruption or traumatic herpes)

In addition to the less common clinical syndromes outlined above and apart from the clinically typical lesions of herpes simplex, any recurrent appearance of grouped vesicles, or their mucous membrane counterpart anywhere on the body, should arouse the suspicion that

the virus of herpes simplex is the causal agent, and appropriate steps should be taken to confirm or refute this.

B. THE VIRUS AND ITS PATHOLOGIC LESIONS

The virus of herpes simplex has the following characteristics:

1. *Size.* 100 to 150 mμ in diameter.[13]

2. *Filtration.* When suspended in broth it can pass a Berkefeld V filter candle.[14] It can also pass through collodion membranes of porosity greater, but not less, than 200 mμ. From this, the size shown above was calculated.[13]

3. *Centrifugation.* It can be sedimented at 5,000 r p m. in 2 to 2½ hours.[15] Centrifugation at 15,000 r.p.m. for 1 hour in an angle high-speed centrifuge (Sorvall) at 4° C. sedimented 96 to 98 per cent of the virus from amniotic fluids containing from 10 to 30,000,000 particles/1 ml.[16]

4. *Electrical reaction.* By a simple filter paper method the iso-electric point was found to be between 7.2 and 7.6, the virus being negatively charged between pH 7.6 and 8 0, and positively charged between pH 6 6 and 7.2; [17] whereas by electrophoretic methods it was found that the particles were negatively charged from pH 6 8 to 7.8.[18]

5. *Visibility.* In highly purified preparations elementary bodylike objects can be demonstrated by dark field illumination or by staining with Giemsa [19] or Victoria blue.[20]

6. *Susceptible hosts.* Man, rabbit, mouse, guinea pig, cotton rat, hamster, and the embryonated hen's egg are susceptible.

7. *Pathologic characteristics.* There are two histologic features that are characteristic of but not peculiar to herpes simplex virus infections (*a*) The presence of type A intranuclear inclusion bodies which are found in all types of infected tissue. These are defined by Cowdry [21] as amorphous granular or rounded intranuclear masses, with an eosinophilic staining reaction The nucleus is severely disorganized, with the basichromatin collected at the nuclear membrane. These inclusion bodies may be seen at all stages of development, from small to large forms. There is usually severe tissue reaction in the neighborhood of type A inclusions. (*b*) The presence of "ballooning cells" which are found in epithelial tissue only.

A more detailed description of the pathologic picture seen in the various tissues infected by the virus is given later in connection with the technics involved in the proper diagnosis of this infection.

II. METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

A. SPECIAL PRECAUTIONS

The virus is not dangerous for most of those working with it since most adults have neutralizing antibodies against the virus. It is potentially dangerous for persons without neutralizing antibodies and for those with atopic eczema. As a rule, no special precautions need be observed.

B. SOURCES OF MATERIAL AND COLLECTION OF SPECIMENS

The shortest possible time should be allowed to elapse between the collection and the inoculation of the specimen There seems no doubt that delay, under conditions of ordinary refrigeration or even with freezing, may result in partial or complete loss of the virus.

Since the virus causes a variety of clinical symptoms, several types of material will be studied for the presence of virus, each of which must be handled appropriately Many specimens will need treatment for the removal of bacterial contaminants before inoculation. The following technic has been found useful in most cases:

Allow the material to be inoculated to stand for one-half hour at room temperature, with one-tenth its volume of the following mixture

Penicillin	5,000 units/ml
Streptomycin	1,000 micrograms/ml

If fungus contamination is encountered or suspected, the addition of crystal violet to the above mixture to a dilution of 1:5,000 makes it fungicidal but does not otherwise alter its properties in regard to the virus or the egg membrane The final dilutions are

Penicillin	500 units/ml
Streptomycin	100 micrograms/ml.
and, if present	
Crystal violet	1:50,000

This mixture does not kill the virus nor is it toxic to the chorioallantoic membrane When crystal violet is added to a mixture of penicillin and streptomycin, a fine precipitate forms This is apparently the result of a compound of crystal violet (base) and penicillin (acid) This "combination" does not affect the activity of the mixture and can be ignored

1 *Swabs from ulcers of mouth, eye, or genitalia.* The swab should

preferably be moistened with a suitable solution * but can be used dry. It should be firmly applied to the ulcer and then placed in a sterile tube containing a few drops, to 0.5 ml, of the solution chosen. (Physiologic saline solution should be used only if no other solution is available—see under "Preservation"—and if the swab is going to be inoculated at once.) If not to be inoculated at once, the tube should be quick-frozen and placed in the freezing compartment of a refrigerator, or in a deep-freeze compartment, at −15° to −20° C. If facilities for freezing are not available, an equal amount of sterile neutral glycerol should be added to the wetting solution, and the tube should be kept in a refrigerator until used.

2. *Vesicle fluid.* The fluid should be collected from a fresh vesicle. This is most efficiently done by using 1 or 2 fine glass capillary tubes. If experimental animals are immediately available, the contents of the tubes can be expressed onto the prepared site (see below) by means of a small rubber bulb such as is used with smallpox vaccine vials. It should be noted that vesicle fluid clots in the capillaries on standing. If the contents cannot be expressed by blowing, the glass tubes can be pulverized in a little diluent and, after centrifuging out the debris, the supernatant can be used for inoculation. Also, fluid can be withdrawn by means of a fine needle (26 or 27 gauge) and syringe. If sufficient material is not thus obtainable the vesicle can be washed out with gelatine-saline and the washings inoculated. If animals are not immediately available, the material must be preserved by freezing, either in the capillaries sealed at the ends or in a small test tube.

3. *Saliva.* This should be collected in a glass receptable and used at once or transferred to a test tube and kept frozen.

4. *Cerebrospinal fluid.* This should be collected with the usual aseptic precautions and either used immediately or preserved frozen.

5. *Brain or spinal cord.* Portions of the brain or spinal cord should be taken at autopsy, using all possible precautions to minimize bacterial contamination. Some portions should be placed in sterile neutral 50 per cent glycerol and saline, and others should be preserved by freez-

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* Suitable diluents

a) Buffered gelatin saline
   Buffer Solution A—0.2 M Na_2HPO_4 (mol. wt. 141.98)
   Buffer Solution B—0.2 M KH_2PO_4 (mol. wt. 136.24)
   Add 67.0 ml of Solution A and 33.0 ml of Solution B to 1900 ml of physiologic saline (0.85%)
   Add 10 gms of bacto-gelatine (Difco) (0.5%) to buffered saline above and heat to dissolve
b) Nutrient broth. This may cause excessive reaction in mouse brain or on the chorioallantoic membrane of eggs if used full strength.
c) Physiologic saline + 10% normal rabbit serum.

ing. It has been shown [22] that encephalitogenic strains of the virus are often more easily demonstrable after the material has been stored in glycerol.

6. *Blood* This should be removed in the usual way, allowed to clot, and the serum separated using aseptic precautions The clot can be preserved in 50 per cent glycerol or ground with sterile alundum (90 mesh) in a small quantity of suitable diluent.* After centrifugation at 2,000 r p m. for 10 minutes to remove the coarse debris, the supernatant is either inoculated immediately or kept frozen. If specimens must be shipped, they should be preserved in 50 per cent neutral glycerol, in rubber-stoppered tubes, and sent by the quickest postal or air express routes.

C. ANIMAL INOCULATION

There are two experimental animals which appear to be equally well suited for primary isolation of the herpes virus, the rabbit and the embryonated hen's egg. The virus can be adapted to the white mouse with moderate ease after primary isolation, also to the guinea pig, hamster, and cotton rat, and can sometimes be isolated in these hosts on primary inoculation

1. *Rabbit* There are two methods of inoculation:

a Corneal route This has long been known to be suitable for isolation of the herpes virus [1] from all superficial or bacterially contaminated tissues without preliminary antibiotic treatment. The technic is as follows·

Under ether or nembutal anesthesia, protrude one eyeball of the rabbit by firm pressure against the lower lid with the butt end of a pair of forceps Steadying the eye by this means, scarify the cornea thoroughly with vertical and horizontal strokes of a von Graefe knife or other suitable instrument, taking care that the incisions actually go through the epithelial layer Turn the rabbit over and repeat the procedure on the other cornea Take the infected swab and, with the eyes back in their sockets, rub it firmly over each scarified cornea and well down under the nictitating membrane If fluid from a capillary tube or needle is being inoculated, place the material on the cornea and massage it in with the eyelids

The rabbit should be observed daily for the development of a keratoconjunctivitis If the test is negative, the rabbit may show slight conjunctival injection for 24 to 48 hours after inoculation but often shows no evidence of trauma.

* See footnote on p. 248

If positive, a characteristic keratoconjunctivitis can occur from 12 hours to 7 days after inoculation The first signs are an injection of the conjunctiva, a slight steaminess of the cornea, which is due to the formation of numerous vesicles along the scratch marks as can be seen with a suitable magnification, and some watery exudate Within another 24 hours, the exudate becomes purulent and photophobia becomes evident The nictitating membrane is injected and swollen Typical histologic lesions in the cornea can be seen within 24 hours after the onset of signs of infection (see below) The conjunctival exudate has been described as largely polymorphonuclear[23] in character, but a high percentage of mononuclear cells is seen in early exudates. Such exudates are bacteriologically sterile and can be used for passage to the cornea of another rabbit or for intracerebral inoculation into rabbits or mice Perhaps a more reliable method for intracerebral passage of the virus is to snip off, under anesthesia, the nictitating membrane at the height of the infection, wash it in sterile saline, grind it with alundum and diluent and, after centrifuging at 2,000 r p m , inoculate the supernatant intracerebrally into the passage animal[24]

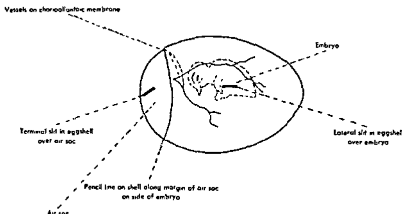
b. Intracerebral route. Aseptic tissues, for example, emulsified blood clot, brain tissue, or cerebrospinal fluid, can be injected intracerebrally in 0.25 ml amounts. Preliminary temperatures should be taken to avoid unsuitable animals that are already running a fever (temperatures of over 104° F are considered significant) If virus is present the rabbit will develop a fever, usually within 24 to 72 hours after inoculation Several possible courses may then be followed. (1) The fever may last a few days without other symptoms, and the rabbit may recover. Such a rabbit will usually develop antibodies against the virus and resist a challenge dose of a known herpes virus after 3 to 4 weeks (2) The fever may persist over 105° F. for several days without any other observable symptoms and the rabbit may die suddenly (3) The rabbit may develop signs of encephalitis, such as tremors, weakness of limbs, or the pulling of the head and neck to one side and moving in circles to that side For passage the animals should be sacrificed, the brain removed aseptically, ground in suitable diluent,* centrifuged to remove coarse particles, and the supernatant inoculated intracerebrally into another rabbit or other passage animal

2 Embryonated eggs The chorioallantoic membrane has been shown by a number of workers to be highly susceptible to this virus The most extensive discussion of technics is given in Beveridge and Burnet's recent report on the use of the chick embryo for growing viruses.[25] The following technics have been used very satisfactorily in this laboratory·

* See footnote on p 248

a All bacterially contaminated or potentially contaminated material should be treated with the antibiotic mixture given above.

b Ten-to 13-day-old embryonated eggs, in which the chorioallantoic membrane is well developed, should be used and prepared as follows: (1) candle the egg and pencil in the margin of the air sac on the side of the shell corresponding to the embryo (see sketch); (2) cleanse the shell over the embryo with 70 per cent alcohol, (3) with a grinder



and a carborundum disc, one inch in diameter, carefully grind a broad slit, approximately one-eighth to one-quarter inch long in the long axis of the shell, down to but not through the shell membrane Wetting the slit with an alcohol sponge will blacken the membrane when the shell has been perforated Grind another similar slit over the center of the air sac (4) With the egg lying horizontally in a suitable holder,* resterilize with alcohol and place a drop of saline over the lateral slit with a syringe of sterile saline and a 22-gauge needle, then, with a gentle rocking motion insert the needle, bevel up and almost parallel to the shell, through the saline in such a way as to pierce the shell membrane Withdraw the needle When the shell membrane has been pierced the drop of saline will slowly seep into the egg between the shell membrane and the chorioallantoic membrane; (5) with a pin or needle, pierce the shell membrane over the air sac through the terminal

* A convenient holder is a fiberboard egg tray which holds 30 eggs These come in nests of 140 trays and bear the trade name of "News Filler Flats" They can be procured from the Keyes Fibre Company, Waterville, Maine

slit; (6) with the egg over the candling box apply suction to the terminal hole over the air sac, watching a "false" air sac form by the entrance of the air through the slit placed over the embryo. A disappearance from view of the clearly marked blood vessels indicates the entrance of air between the shell membrane and the chorioallantoic membrane. If the membrane does not drop on the first trial it is advisable to make another slit over the region of the embryo and to try again, placing a finger over the first slit while sucking out the air from the air sac. (7) Take up 0.05 ml. of the material to be injected in a ½ or ¼ ml. tuberculin syringe and, using a 22-gauge needle, inject it into the false air sac over the embryo; (8) protect the slit over the embryo by covering loosely with scotch tape. This prevents contamination with gross particles but allows sufficient air entry to maintain the air sac. The slit over the end of the egg does not need sealing. (9) Incubate eggs at 95° to 96° F for 48 to 72 hours; (10) remove from incubator and candle again If the normal air sac has reformed, as it does occasionally, leaving the chorioallantoic membrane up against the shell membrane, remove the scotch tape and suck the air out of the normal sac to reform the false one. (11) Open the eggshell with scissors, outward from the slit and, under direct vision, push down the chorioallantoic membrane from off the shell, taking off the shell so freed Open the egg membrane from below to allow the escape of fluids and yolk This allows the whole of the "dropped" area of the chorioallantoic membrane to be completely exposed Cut around this with scissors. (12) Remove the excised part of the membrane to a petri dish containing 1 per cent formol saline The outer surface of the bottom of the dishes used for this purpose should be blackened with enamel paint The dish can be placed on a black background but painting is far superior (13) Flatten out the excised membrane and examine under a good light with a 2× lens. (14) if virus is present, the membrane will either show a marked generalized thickening over the inoculated area or a mass of confluent grayish white plaques, or numerous small discrete plaques varying in size from 0 5 to 2 0 mm. depending on the amount of virus present

Beveridge and Burnet[254] suggest the following general criteria for differentiating specific from nonspecific lesions seen on egg membranes The specific lesion tends to have (1) a circular shape, (2) a more opaque central part, which may become necrotic, and (3) a surrounding haze due to mesodermal infiltration with inflammatory cells This description fits the plaques of vaccinia virus perfectly In herpes, however, these characteristics are modified in that the plaques tend to be oval or

actually have a tail, and neither necrosis nor the mesodermal bare is prominent. The plaques give the impression of being very superficial and can in fact be scraped off the membrane On primary isolation they may be very small and must be searched for with magnification in a good light For passage the chorioallantoic membrane is removed according to the above technic, only without the addition of formalin, and ground with alundum and gelatin saline After centrifugation at 1,500 r p m to remove coarse particles, the supernatant fluid is inoculated onto the chorioallantoic membrane of 10- to 13-day eggs as described above It may take several passages before typical plaques develop, and any membrane which shows suggestive plaques or only edema, even in the presence of obvious nonspecific irritation (thickening and irregular opacities along the blood vessels) should be passed on to other eggs at least once

3. *Other animals.* When the virus is passaged to hosts other than the two just described, which are perhaps the most useful for direct inoculation, the following disease patterns may be seen:

a Mice After intracerebral inoculation, the animals on primary isolation may not show symptoms for 5 to 7 days but, in a well-adapted strain, they may show symptoms as early as 24 hours or, more usually, 2 to 4 days after inoculation Usually the first significant sign is marked jumpiness and hyperactivity on stimulation A knock on the cage sets them leaping, convulsions occur after spinning by the tail, then the fur becomes roughened, the gait spastic, and activity lessened Death often occurs after a tetanic convulsion with the front and hind legs extended backwards, a position similar to that seen in infection with lymphocytic choriomeningitis However, they are often found dead without showing any typical position After intraperitoneal inoculation, the incubation period is longer and the mortality is usually lower than after intracerebral inoculation The animals appear sluggish, with roughened fur Typical convulsive seizures are not so common as after intracerebral inoculation, the mice dying rather unexpectedly Virus is present in the brain after intraperitoneal inoculation[9b]

b Guinea pigs Apart from keratoconjunctivitis after corneal inoculation, which does not differ essentially from that of the rabbit, dermal strains of the virus can produce intracutaneous and footpad lesions. About 2 days after intracutaneous injection of virus, red papular lesions develop, which may vesiculate and then subside after 2 to 3 days The inoculated footpads become inflamed and swollen on the second day, the reaction gradually subsiding in the course of a few days After intracerebral inoculation, the animals may develop fever and occasionally convulsions[9b]

c. Hamsters. After intracerebral inoculation, these animals show chiefly hyperactivity, then sluggishness, muscular inco-ordination, tremors, and, in some instances, paralysis and death.[9b]

d. Cotton rats. This species has been reported as being very susceptible to the virus by the intranasal route as well as by the intracerebral route.[26]

D. PATHOLOGIC SPECIMENS

These may be obtained by biopsy of characteristic superficial lesions, such as herpetic vesicles from autopsy material, particularly the brains of patients who died of encephalitis, and from typical lesions in the eye and brain of the rabbit, the brain of the mouse or other infected animals, and the plaques on the chorioallantoic membrane. The diagnostic finding in herpes virus-infected tissue is the presence of the *type A intranuclear inclusions already described (page 246). These are* commonly known as Lipschütz bodies. They are characteristically demonstrable in the infected cornea of the rabbit. In order to see them it is important to take the rabbit's eye out from 12 to 24 hours after the appearance of signs of conjunctivitis and before conjunctival exudate has become frankly purulent. After this time, the epithelial cells in which the inclusions occur have mostly sloughed off. It is important to note that the time for section must be judged by the *appearance of the cornea and the time after the onset of symptoms and* not from the time of inoculation, since the incubation period will vary with viruses of different pathogenicity. Typical inclusion bodies can also be seen in the brains of rabbits, mice, and human beings, in the skin and mucous membranes of man with the natural infection, and in the dermal lesions of animals They can also be seen in lesions of the egg membrane when examined at 24 hours. Anderson[27] used their presence to determine the presence of virus, but Beveridge and Burnet[25b] point out that they differ somewhat from those in the rabbit cornea.

Apart from the inclusion bodies, the histologic reaction varies with the tissue being examined but is, in general, of an acute inflammatory nature The characteristic pathologic lesion of herpes is the vesicle which develops in its complete form in the skin, and in a modified form in the mucous membranes In the skin, the lesion is histologically indistinguishable from those of herpes zoster and varicella In the fully developed vesicle there appears to have been a proliferation of the cells of the basal layer of the epidermis These undergo "ballooning degeneration" and inclusion bodies can be found in their nuclei These are best seen at the edge of the lesion Colliquative degeneration takes place in the cells of the lower epidermis extending down to, *but not involving, the corium,* which

explains the absence of scarring The resultant vesicle lies deep in the epidermis roofed over with degenerating prickle cells and imperfectly keratinized horn cells The vesicle fluid contains fibrin, enmeshed in which are detached swollen epidermal cells, some of them grouped together as giant cells In many of these intranuclear inclusion bodies can be found Leukocytes are also present in the fluid In the corium there is a pronounced inflammatory reaction with dilated capillaries and a polymorphonuclear cell infiltration The vesicle in the mouth resembles the above but because of maceration the vesicle ruptures early leaving the space filled with fibrin [28, 14]

In the rabbit cornea there is an early proliferation of the epithelial cells in the neighborhood of the scratch marks In the center of the plaque so formed, necrosis occurs with eventual sloughing of the proliferated cells The inclusions are best seen in the cells at the margin of the scratches or of the necrotic area In the *substantia propria*, there is considerable congestion and acute inflammatory reaction accompanied by polymorphonuclear infiltration [28] It should be noted that the granules of the polymorphonuclear cells of the rabbit tend to be quite eosinophilic and show up red with the hematoxylin and eosin stain and may, therefore, be mistaken at first glance for possible inclusion bodies In the rabbit brain, DaFano and Perdrau [29] describe a chronic inflammatory lesion occurring in partially immunized rabbits In the human brain, the following picture is described by Zarafonetis [30]

"Intense vascular congestion was uniformly present in the central nervous system, with many areas of small ecchymoses and petechiae in both the brain substance and subarachnoid area A moderately severe focal meningitis, with infiltrations of lymphocytes and large mononuclear cells generally occurring about vessels, was found over the cerebral hemispheres and the cerebellum and about the spinal cord

"The most striking lesions in the brain substance were found in the cortex and subcortical white matter, particularly in the vicinity of the area of softening in the left temporal lobe Perivascular cuffs composed of lymphocytes in the Virchow-Robin spaces were present throughout this region and were occasionally encountered in the midbrain and pons The endothelial cells lining such vessels were frequently swollen Proliferated glial cells were numerous in the tissue about the cuffed vessels Within the area of encephalomalacia in the left temporal region there were numerous small hemorrhages, as well as foci of spongy necrosis and large collections of 'Gitterzellen' among which a moderate number of polymorphonuclear leukocytes were visible

"Intranuclear inclusions of the herpetic type were present in profusion in certain portions of the cerebrum These appeared as bright pink, homogeneous or granular structures occupying most of the space within the nuclei of cells in sections stained with phloxine and methylene blue or by Giemsa's technic. Occasionally the characteristic picture of an A type intranuclear inclusion was seen, i e, a large pink body surrounded by a clear zone in which the blue nucleolus lies close to the dark nuclear membrane which is thickened because of margination of chromatin Most of the acidophilic inclusions were found in glial cells but a few nerve cells were similarly affected"

Inclusion bodies in the chorioallantoic membrane are somewhat different from those just described but are easily recognizable [31]

c. Hamsters. After intracerebral inoculation, these animals show chiefly hyperactivity, then sluggishness, muscular inco-ordination, tremors, and, in some instances, paralysis and death.[9b]

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F. OTHER VIRUSES TO BE RULED OUT

Four viruses might possibly be confused with the herpes virus:

1 *Vaccinia.* This virus causes a vesicular eruption on the atopic eczematous skin (eczema vaccinatum) which is indistinguishable from eczema herpeticum. It causes plaques on the chorioallantoic membrane of eggs which are larger and more necrotic than herpes but which can only be distinguished for certain by finding typical inclusion bodies or by a specific neutralization test. It also causes a keratoconjunctivitis in rabbits which, however, can be distinguished by the presence of the cytoplasmic Guarnieri bodies of vaccinia in the histologic section.

2. *The virus of epidemic keratoconjunctivitis.*[32] This causes a conjunctivitis sometimes indistinguishable from that caused by the virus of herpes simplex. The virus is thought by some workers[3] to bear an immunologic relationship to that of herpes simplex. It may be distinguished by its size, 25 to 50 mμ, as compared with that of herpes simplex, 100 to 150 mμ It causes a keratoconjunctivitis only irregularly in rabbits and fails to infect the guinea pig. It can be distinguished by suitable neutralization tests.

3. *B virus.*[33] This causes an ascending myelitis which might simulate a central nervous system infection with the herpes virus. The one case reported, however, did not clinically resemble any of the four fatal cases of herpes encephalitis which have been reported The two viruses are very closely related and can only be distinguished by careful cross-immunity tests in rabbits and cross-neutralization tests with immune animal sera. *N.B.* The virus of pseudorabies is immunologically related to both herpes and B virus[31] Since, however, this virus only infects animals, it should not cause confusion with human herpes

4 *The virus isolated by Buddingh and Dodd*[34] *from newborns manifesting diarrhea with or without an ulcerative stomatitis.* This virus does not cause a typical keratoconjunctivitis in the rabbit but more usually an intense iritis, and no inclusion bodies have been observed. This virus has as yet been too incompletely studied for more details of the differentiation to be given.

G. IDENTIFICATION OF THE VIRUS

This can be done in two ways:

1. *Immunity test* By challenging herpes-immune animals and normal controls with the virus under consideration If the virus is herpes, the normal animals will come down with typical disease and probably die, but the immune animals will show little, if any, reaction.

E. STAINING

There are several stains that have reportedly been used with success for showing up the inclusion bodies so characteristic of the disease. The final stain adopted in any one laboratory will depend largely on the experience of that laboratory in the use of various stains. Hematoxylin and eosin is probably the most widely used stain, although some authors [9c] report better results with phloxin and methylene blue. Giemsa is also often used. Proper adjustment of the acid and basic stains is important in bringing out the contrast of the inclusion body in the nucleus. In some reports it is noted that the inclusion body approaches a magenta more nearly than the pink. These differences may depend on the staining technic. In this laboratory hematoxylin and eosin have been quite successful but perhaps the best results have been obtained by Masson's modification of Mann's stain, especially in sections of rabbit brain. Because somewhat less frequently used than the hematoxylin and eosin stain, the necessary points in technic are here given:

1 Fix tissues in 5 per cent formol Zenkers or Helly solution for not longer than 24 hours.

2. Wash, embed, and mount in the usual way.

3 Remove paraffin and hydrate in the routine manner.

4. Place slides in Lugol's solution for 30 minutes or longer to remove precipitate.

5 Wash in running water for 30 minutes or until sections are white.

6. Wash with 95 per cent alcohol to remove excess Lugol's solution, then rinse with tap water.

7 Put the sections in the following stain for 2 to 3 hours until they turn pink (In some cases better results are obtained by heating the stains at 56° C during the period)

Erythrosin (Grubler or Krall)	0 2 gms
Orange G	1 0 gms
Distilled water	100 0 gms

8 Wash in distilled water

9 Place, *for not more than 2 minutes*, in a 1 per cent solution of toluidine blue.

10 Wash in distilled water

11 Differentiate in a dilute acetic acid (1,500) until the sections, under a microscope, have a pinkish cast but the nuclei are still blue

12 For dehydration, dip a few times in absolute alcohol, repeat with blotting, then repeat in 1 per cent alcoholic solution of acetic acid, then repeat again in absolute alcohol, using frequent blotting to hasten dehydration

13. Clear in creosote xylene and two changes of xylene, 5 minutes each (minimum).

14. Mount in Canada balsam in the usual way.

2. *Neutralization test.* By specific neutralization of the virus by a known herpes-immune or hyperimmune serum.

In order to demonstrate neutralization of the virus by a known specific serum, the strength of the virus in terms of infectious units must be determined by titration. The end point of a suspected virus mixed with specific serum can then be compared with the end point of the suspected virus in the presence of normal serum. Since the technics of titration and neutralization overlap, that of titration will be described here whereas that of neutralization, either for suspected virus by a specific antiserum or a known herpes virus by a suspected immune serum, will be described under the section dealing with serologic diagnosis of the disease.

H. TITRATION OF VIRUS

This can be done in two ways: in mice and in eggs

1. *In mice.* The brain of a mouse with suspected herpes infection or portions of brains of several infected mice are removed aseptically, weighed, and then ground up with a suitable diluent * into a 20 per cent emulsion; this is centrifuged at 2,000 r p m. for 10 minutes and the supernatant diluted serially in 10-fold dilutions † 10^{0}–10^{-6}. With a ¼ ml. tuberculin syringe and a 27-gauge ¼" needle, take up 0.25 ml. of the 10^{-6} dilution. Under light ether anesthesia inoculate 4 to 6 mice intracerebrally, each with 0.03 ml. With the same syringe and needle, repeat the procedure with the lower dilutions in turn.

The mice should be examined daily All mice dying within 24 hours of the inoculation should be discarded from the test as due to accident. Mice dying of the disease show the characteristics described above The number of mice dying or showing symptoms of encephalitis should be recorded each day The test should be kept for 21 days, although if the strain is very potent, 2 weeks may be sufficient At the end of this time, the number of mice that have died or have been typically sick with encephalitis and the number surviving and healthy should be tabulated for each dilution and a cumulative total derived, so that a 50 per cent end point can be calculated according to the formula of Reed and Muench ‡ [89]

2. *In eggs.* Since the virus produces characteristic plaques on the chorioallantoic membrane, this provides a convenient and inexpensive method of titrating the virus. One or more chorioallantoic membranes infected with the herpes simplex are weighed and ground in suitable diluent * with sterile alundum to a concentration of 10 per cent This is centrifuged at 2,000 r.p m for 10 minutes and the supernatant

* See footnote on p 248.

† IN ALL VIRUS TITRATIONS, A SEPARATE PIPETTE SHOULD BE USED FOR EACH DILUTION

‡ See explanatory note on page 259

0.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5

EXPLANATORY NOTE

CALCULATION OF 50 PER CENT END POINT

In *Column 1:* place the dilutions of virus (or serum if such is being examined) from the lowest dilution above to the highest below In *Columns 2 and 3.* note the numbers alive and dead respectively opposite each dilution. In *Columns 4 and 5:* cumulate the survivors and deaths respectively If the virus is being titrated, start the additions in the "survivors" column at the top and the figures in the "death" column at the bottom If a serum is being titrated for neutralizing properties, reverse the order starting the survivor column at the bottom and the death column at the top In the *last column:* compare the deaths and survivors at each dilution and record in terms of per cent deaths It will be obvious that the 50 per cent mortality point lies between two dilutions The exact titer at which 50 per cent end point occurs is calculated by the following formula:

$$\frac{50 - \text{mortality at dilution next below}}{\text{mortality next above} - \text{mortality next below}} = \textit{proportionate distance}$$

Since dilutions are increasing on a log scale, the final reading is obtained as follows

Log of lower dilution + proportionate distance × log of dilution factor = log of end point

This is more easily done graphically

The graph on semilog paper enables the end point to be calculated for any dilution factor Obtain the proportional distance as described Read off the point (on the ordinate) where the proportionate distance (on the abscissa) *crosses* the dilution line The product of this figure and the lower dilution (of the two between which the 50 per cent end point falls) gives the final end point

For example 1. If, in an experiment, the dilution were 2-fold, the 50 per cent end point fell between 16 and 32 and the proportionate distance worked out at 0 75, then the 50 per cent end point would be 1 68 (the point where 0 75 line crossed the 2× dilution line) × 16 (the lower dilution) = 27 The final titer would then be 1 27.

2. If, in an experiment, the dilution were 10-fold, the 50 per cent end point fell between 10^{-4} and 10^{-5} or 1.10,000 and 1.100,000, and the proportionate distance worked out at 0 75, then the 50 per cent end point would be 5 65 (the point where 0 75 line crossed 10× dilution line) × 10,000 (the lower dilution) = 56,500 The final titer would then be 1 56,500

diluted * in decimal steps to 10^{-6} or 10^{-7} in the above diluent. Using the same ¼ or ½ ml. tuberculin syringe and a 22-gauge needle, 0.05 ml. of each dilution, starting with the highest, is inoculated onto the chorioallantoic membrane of each of four 10- to 13-day-old eggs, all of which are harvested after 48 hours as described below. In the lower dilutions, the plaques may not be distinguishable, the whole membrane being opaque, boggy, and edematous; with higher dilutions, however, discrete plaques will appear in numbers that can be accurately counted, until an end point is reached beyond which no plaques are visible and the membranes have their normal thin, glistening appearance. The reproducibility of the end point has been studied by Beveridge and Brunet.[25c] They calculate that with their strain of herpes simplex four membranes per inoculation give results within ± 50 per cent of the mean. In this laboratory also this appears to hold true.

There are certain difficulties encountered in making the counts In some membranes with higher plaque counts, the inoculum does not get evenly distributed and, therefore, there is a fused collection of plaques in the center which is uncountable This tends to disturb the accuracy of the final average count. However, if grossly distorted membranes are discarded and the rest averaged, the results appear to be accurate within the limits of the method A recent modification, suggested by Beveridge and Burnet[25e] weights the counts on good membranes by using them twice in obtaining an average whereas the counts on poor membranes are used only once. Comparison of the average plaque counts obtained by the two methods, however, reveals as a rule such a small difference that this modification will probably be rarely needed

In working out the final titer of the virus from the membrane inoculations, the size of the inoculum and the dilution of the original material must be considered as well as the average number of plaques at a certain dilution; for example, *if there is an average of 20 plaques at a dilution of 10^{-4}, then the amount* of virus in 1 0 ml of the original is 20 (number of plaques) × 10,000 (dilution) × 20 (fraction of 1.0 ml inoculated) = 4×10^6 = virus titer (concentration of infectious units/ml).

The 50 per cent end point titer of mouse brain virus in mice is usually not reported as exceeding 10^{-4}, but in strains that are well egg-adapted, virus concentrations in eggs of 10^8 or 10^9 infectious units/ml may be obtained

I. PRESERVATION AND DESTRUCTION

There appears to be some difference in the ease of preservation between virus in brain material and virus in egg material. Most of the information in the literature pertains to the former. This can be preserved in 50 per cent glycerol at 4°–8° C. for 6 months and for a longer

* See footnote on page 248.

time (18 months) when desiccated from the frozen state.[a] It can be maintained frozen in the moist state at −20° C. for many months but is better preserved at −70° C.[a] As egg material, however, only 1 out of 7 specimens were recoverable after 7 months in a dry-ice box. This deterioration is apparently not influenced by quick or slow freezing or by any of the three recommended diluents. When frozen from the moist state and kept at −20° C. the activity of the egg virus should not be relied upon for more than 7 to 10 days.[18] The virus in any form deteriorates rapidly in physiological saline, but this can be overcome by buffering the saline to a pH of 7.2 and adding 10 per cent rabbit serum[17] or one-half per cent gelatin.[16] It also keeps better in nutrient broth. A low oxygen tension, such as attained by adding cysteine to the preserving fluid,[17] also helps to preserve the virus. The virus is destroyed by bile[22, 40] and a number of other chemicals, particularly alcohol, chloroform, and ether in the wet state, although in the dry state the virus can resist the action of both alcohol and ether.[28] Zephiran chloride (alkyl dimethyl benzyl ammonium chloride), which is a relatively nontoxic substance and easily tolerated by animal and human tissues, reduced the LD_{50} titer of two strains of herpes virus in mice, after 30 minutes incubation at room temperature at a final concentration of 1:5,000, from 10^{-4} to 10^{0} and from 10^{-5} to 10^{-1} respectively.[14] Gentian violet is relatively harmless to the virus. An infected rabbit brain emulsion, exposed to 1 per cent gentian violet for 18 hours at 4° to 8° C. and then washed free of the dye still contained sufficient virus to infect a rabbit on intracerebral injection.[29] However, if an infected chorioallantoic membrane emulsion is exposed to 1:5,000 gentian violet for 30 minutes and the whole mixture is inoculated onto chorioallantoic membranes of other eggs, the virus will have lost 95 to 99 per cent of its potency. No reduction of potency was found when the gentian violet concentration was reduced to 1:50,000.[18] The virus is also comparatively resistant to phenol; it survives contact with 1 per cent solution for 15 minutes but is destroyed after contact for 3 hours.[22] It is destroyed by exposure to 1:1,000 potassium permanganate solution for 1 hour at room temperature.[56] The effect of temperature varies with the state of the virus. In the moist state it is destroyed at 52° C. for 30 minutes; in the desiccated state it resists 90° C. for 30 minutes

[a] When storing the virus in a dry-ice cabinet it is important to use glass-sealed ampules. The use of rubber stoppers allows entrance of CO_2 into the vials which, in some cases, causes deterioration of virus.

but is destroyed by heating at 100° C. for 30 minutes.[36] It is potent after 6 to 8 hours but is destroyed after 24 hours at 37°.[1c] The observations in this section were made on virus in the form of tissue emulsion, unless otherwise indicated.

III. SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS OF DISEASE

A. COLLECTION OF MATERIAL

Blood should be collected shortly after the onset of a clinical syndrome suspected of being a herpetic infection, allowed to clot, and the serum withdrawn aseptically. This should be repeated at 2- to 4-week intervals. These two sera are used for serologic tests to demonstrate the appearance of antibody against herpes or to prove the absence of such a phenomenon. The sera can be used fresh or stored in the refrigerator. They can be shipped in sterile, rubber-stoppered tubes in regulation mailing cartons No preservatives are to be added. For prolonged storage the sera are best preserved frozen.

B. NEUTRALIZATION TEST

1. *In mice.* A satisfactory test is that described by Ruchman *et al*[6] as follows:

Twenty per cent suspensions of infected mouse brain are prepared each time. Decimal dilutions are made in rabbit serum saline * giving concentrations of 1:5, 1.50 . . . etc, up to 1:500,000 or 1:5,000,000. These, when added to equal amounts (0 15 cc.) of undiluted test sera, give final concentrations of 1:10, 1:100 . . . etc, up to 1·1,000,000 or 1:10,000,000. The mixtures of virus dilution and serum are incubated in a water bath for 2 hours at 37° C. At the end of this time the mixtures are injected intracerebrally into groups of 4 or 5 mice which are observed daily over a period of 21 days for central nervous system signs and death. LD_{50} titers can be calculated according to the method of Reed and Muench[38] and neutralization indexes can be determined by the method described in *Laboratory Methods of the United States Army*[39] as follows:

The neutralization index of a serum can be calculated as follows

Log of LD_{50} control — log LD_{50} titer of serum = X

Antilog of X = neutralization index

On this basis, the following criteria are adopted·

Neutralization index 1–9	= negative
Neutralization index 10–49	= equivocal
Neutralization index 50 or more	= positive

* See footnote on page 248

2. *In eggs.* The technic of Burnet and Lush[40] can be summarized as follows:

Sera to be used are inactivated at 56° C. for 20 minutes. Equal volumes of undiluted serum and suitably diluted virus are mixed and left in a refrigerator for 1½ to 2 hours Then 0.05 ml of the mixture is dropped on the chorioallantoic membrane of 12-day-old eggs These are incubated for 40 hours and a plaque count made. For an unknown serum, 4 eggs are inoculated with a mixture of undiluted serum and undiluted virus, and 4 with undiluted serum and virus diluted 1:5,000. Results are expressed as the percentage of foci which appear in terms of the count obtained, with a control of normal serum and 1:5,000 dilution of virus.*

In their series of 126, all but 3 were less than 0.1 per cent or between 30 and 100 per cent of the control This method provides a suitable technic for distinguishing between positively neutralizing sera, in which the plaque count is 0.1 per cent or less, and negatively neutralizing sera, in which the plaque count is more than 30 per cent of the control In addition, all sera tested against the same control can be compared in terms of percentage reduction of plaques

In this laboratory the following technic has been found useful in comparing the neutralizing activity of one serum with another, such as the acute and convalescent sera from one patient. A concentration of virus is chosen which will give an easily determined number of plaques (± 100) on the membrane This is incubated with an equal quantity of the serum to be tested and a control serum. The sera are used unheated. The test human serum is used undiluted, and diluted 1:16. The control normal rabbit serum is used only diluted 1:16, since undiluted rabbit serum is found at times to decrease the plaque count Incubation is carried out for 1½ hours at 8° C. The exact time is not important since neutralization apparently takes place very quickly (less than one minute) in this virus-serum combination.[41] 0.05 ml of each mixture is then inoculated onto the chorioallantoic membrane of each of four 10- to 13-day eggs After 40 hours' incubation at 96° F, the membranes are removed and counted as described above. Since the titration of the virus is accurate to ± 50 per cent, the reduction by more than that amount of the number of plaques by the test serum as compared with the control indicates the presence of antibodies[16]

C. COMPLEMENT FIXATION TEST

Up to the present, this test has been of little value in the diagnosis of herpes. Myers and Chapman,[42] using a variety of antigens, were

* The dilution of 1:5,000 happened to be the dilution that gave a number of plaques sufficiently low to count accurately in the experiment cited. Any suitable dilution can be used.

not able to demonstrate a complement fixation test for herpes although, using the same methods, they could for vaccinia and virus III. They quote the literature listing a number of authors who could not demonstrate complement-fixing antibodies and four groups of authors who could. Of the successful methods, the method of Bedson and Bland,[43] used also by Brain in Bedson's laboratory,[44] seems to be the most satisfactory. Bedson and Bland's technic is described as follows:

Antigens were made from the herpes-infected footpads of guinea pigs. The tissue was ground in M/50 phosphate buffer pH 7.6, and made up to 5 per cent or 10 per cent, centrifuged to remove coarse particles, and used unheated This antigen was rarely anticomplementary. The antigen could largely be destroyed by boiling for 30 minutes.

Hyperimmune guinea pig sera were prepared by immunizing animals by 6 to 8 intraperitoneal injections of 1 to 2 ml. of 10 per cent virus. "These sera, if comparatively recent, have been used unheated; to heat them renders them anticomplementary." Some sera became anticomplementary on prolonged storage, but this property could be removed by heating such old sera at 56° for 10 minutes. Rabbit sera were unsatisfactory because many were anticomplementary, and this property could not be removed by heating.

The test was done as follows:

Serum to be tested .. ..0.1 ml.
Antigen ....0.1 ml.
Complement ...1 to 2 units (= 2–4 m.h d.)
Saline ....to 0 5 ml

were mixed and allowed to fix at room temperature for 2 to 3 hours (Fixation at 8 to 10° C. overnight led to more nonspecific fixation.) Sensitized sheep cells were prepared by adding 5 lytic doses of amboceptor to 5 per cent washed sheep red cell suspension After the period of fixation, 0 2 ml of sensitized sheep cells was added, and saline to a final volume of 1.0 ml. The whole was incubated at 37° for 30 minutes. The usual serum and antigen controls to test for anticomplementary activity were set up as were controls using tissue from normal guinea pig pads and, if necessary, tissue from vaccinia virus infected pads.

D. SKIN TEST

Nagler[45] used amniotic fluid containing herpes virus at a titer of 10^7 virus particles per 1 ml. of fluid and heated at 65° C. as a skin test antigen. The protein content of this injected amniotic fluid was

approximately 0 012 per cent. The test was performed on the skin of the forearm by intracutaneous injection of 0 1 ml of a 1 50 dilution of the fluid. In 15 adults who gave a history of herpes and had neutralizing antibodies in their serum, this produced an erythema of 5 to 14 mm in 24 hours, which sometimes lasted for 48 hours, but might fade by that time There was no reaction to the normal amniotic fluid with a protein content of 0 005 per cent used as a control In 15 adults who gave no history of herpes and whose blood was negative for neutralizing antibodies, the skin test was negative.

If such a skin test antigen or another of proved specificity were available, the development of a positive skin test in a patient after an illness of suspected herpetic etiology would support the diagnosis.

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YELLOW FEVER*

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I ISOLATION AND IDENTIFICATION OF THE VIRUS
- A Isolation of Virus from Patient's Blood
  - 1 Monkeys
  - 2 Mice
- B Demonstration of Specific Lesions
  - 1 Human cases
- C Demonstration of Increasing Antibody Titer in Convalescence
- D Application of These Methods to Animal Studies

II SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS OF DISEASE
- A Intraperitoneal Protection Test
  - 1 Adult mouse test (Lloyd and Sawyer)
    - a Specimens to be examined
    - b Preparation of virus
    - c Preparation of serum-virus mixtures
    - d The starch solution
    - e Controls
    - f Inoculation of the mice
    - g Inspection and recording
    - h Interpretation of the results
  - 2 Adult mouse test (Smithburn)
  - 3 Young mouse test (Whitman)
    - a Test virus
    - b Serum-virus mixture
    - c Controls
    - d Inoculation of mice
    - e Interpretation of results
- B *Intracerebral Protection Test*
  - 1 Method of Theiler
    - a Procedure
    - b Materials and controls
    - c Interpretation
  - 2 Method of Pasteur Institute, Dakar
    - a Procedure
    - b Interpretation
- C Comment

III REFERENCES

* Because such a large portion of the procedures described in this manuscript was given in quotation, departure was made from the general practice of reducing quotations to small type

THE clinical manifestations in yellow fever are so variable that positive diagnosis is impossible without laboratory assistance. In only a minority of cases is the classical clinical picture encountered. While there are numerous laboratory procedures of value, only those will be considered here which lead to the establishment of the diagnosis, those having presumptive value only will be omitted.

I ISOLATION AND IDENTIFICATION OF THE VIRUS

For the certain diagnosis of yellow fever, three lines of investigation are available:

1. Isolation of yellow fever virus from patient's blood.
2. Demonstration of specific histopathologic lesions at autopsy.
3. Demonstration of a marked rise in specific antibody content of the blood serum during convalescence.

A. ISOLATION OF VIRUS FROM PATIENT'S BLOOD

During the period of circulation of active virus in the patient's blood, the presence of the infective agent may be demonstrated by inoculating whole blood or serum into a susceptible animal. The test animals most commonly used are the rhesus monkey and the white mouse. Many monkeys native to South America and Africa may also be employed satisfactorily if due consideration is paid to the variability in susceptibility of these animals and to the tendency for most of the species to have the disease in a very mild form.

Inoculation of the monkey may be by the subcutaneous or intraperitoneal routes On the other hand, the adult white mouse is relatively insusceptible by these routes so that the suspected serum must be inoculated directly into the brain.

The prospects of successful demonstration of virus are excellent during the first 3 days of the disease, irrespective of the degree of illness, only rarely can virus be shown after the 6th day following the clinical onset There is a marked variability not only in the clinical manifestations of yellow fever but also in the time at which demonstrable virus may appear in the blood stream as well as in the length of time it circulates and the amount present as judged by titration in mice In a suspected case, therefore, it is wise to make daily bleedings for 5 days, beginning as early as possible in the course of the disease It is desirable that at least 10 ml. of blood be taken in order that there will be sufficient serum for subsequent antibody studies.

1. *Monkeys* When rhesus monkeys are employed as test animals, the inoculum should be 1 to 2 ml of the suspected serum. The blood of the animal to be used is examined before inoculation of the virus to make sure that it is free of yellow fever antibodies. A sample of this pre-experimental serum should be preserved for subsequent comparison The rectal temperature of the test animal is taken morning and evening, and elevations above 40° C can be considered as significant In such event the animal should be bled (preferably from the leg vein) and the serum subinoculated into a group of mice (conventionally 6). If the case is one of exceptional importance, then such bleedings may be performed daily, irrespective of evidences of fever. The serum remaining from each bleeding may, if desired, be preserved by freezing and drying or in a low temperature cabinet In the event that the monkey should suffer a fatal illness, autopsy with special attention to the microscopic findings in the liver may confirm the diagnosis at once without waiting for the final evidence in the mice The most satisfactory identification of the virus, however, is in the mouse passage material When the mice become ill, the brains of 1 or 2 whose symptoms are typical may be triturated in 3 0 ml each of 10 per cent normal monkey or human serum in saline diluent Further dilutions of this virus preparation are allowed to react with normal and yellow fever immune sera and the mixtures are tested for surviving virus by inoculation into mice The technic is that of the standard intracerebral protection test. Since it is essential that the neutralization test be specific, it is recommended that the normal and the immune sera be from the same monkey—taken before and after known yellow fever immunization—so that differences other than the presence of yellow fever antibodies will be eliminated.

2 *Mice* Since mice are presumed to be available, it is recommended that a group of these animals be inoculated with each original blood specimen Owing to the high cost of rhesus monkeys, it is usually not possible to devote several of them to a single patient, and where multiple bleedings are obtained in the acute stage of illness, they must of necessity be given to the same animal. The simultaneous inoculation of mice permits more exact information to be obtained with regard to the days of virus circulation

When monkeys are not available, mice can be used alone with good prospect of success But different races of white mice vary markedly in their susceptibility to yellow fever virus, and strains of the virus

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A. *Isolation of Virus from Patient's Blood*

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of profound parenchymatous lesion of the liver and kidneys with a marked tendency to hemorrhages by diapedesis, particularly in the mucosa of the gastro-intestinal tract. Save for these findings and a variable amount of jaundice, there is nothing striking. A positive diagnosis cannot be made from the gross findings, although a diffuse fatty parenchymatous change in the liver may justify a strong suspicion.

While the renal lesion may be as important as that of the liver in causing death, it is the latter that is pathologically diagnostic. Microscopically there is a diffuse, nonpurulent necrosis most prominent in the midzone of the liver lobule and further characterized by discontinuity in the liver cell cords and a tendency for the necrosis to be coagulative, giving rise to the so-called "Councilman bodies" Invariably there is a collar of surviving liver cells about the central vein of the liver lobule Necrosis may and does involve all three zones, and in the fulminating case the process may be so extensive that zonal predilection is lost.

Special fixations and stains are unnecessary. The usual formol fixation with hematoxylin and eosin staining is entirely satisfactory A well-prepared frozen section will enable the diagnosis to be made at once

C. DEMONSTRATION OF INCREASING ANTIBODY TITER IN CONVALESCENCE

When recovery occurs the histologic method cannot of course be applied Furthermore, it happens frequently that the question of possible yellow fever does not arise until several days of illness have elapsed so that attempts to isolate virus are unsuccessful. Diagnosis can still be made by utilization of the fact that the antibody content of the blood, which may be demonstrable with difficulty on the 5th or 6th day of the disease, rises rapidly to reach a maximum in about 3 weeks On comparing the antibody content of acute and convalescent phase sera in yellow fever, there is found a great increase in antibody titer in the second specimen over that in the first.

Serial dilutions of each serum are subjected to a standard protection test The technic may be either an intraperitoneal procedure such as that of Sawyer and Lloyd or the intracerebral method of Theiler. The second has the advantage of requiring a very small amount of serum.

When the first specimen is obtained on the first day or two of the clinical disease, the undiluted serum will have no neutralizing effect against yellow fever virus, and the second specimen may give an end

differ in pathogenicity for mice. These facts have led various workers to mistrust the use of mice for the original isolation of virus. However, my experience has been favorable. In Colombia, in approximately 40 isolation experiments in both rhesus monkeys and mice, when in many cases the virus was from mosquitoes rather than from man, there were only two instances in which evidence of infection was observed in the monkey without manifestation in the corresponding mice. More recently, in Nigeria, West Africa, during the study of an outbreak of yellow fever, 34 isolations of virus from human material were made in mice alone with little difficulty. In most instances, all mice inoculated became ill in a regular manner.

The experience in the laboratory at Entebbe, Uganda, has not been so satisfactory in this respect. In several instances, virus has been obtained by monkey inoculation when none has been noted in the mice inoculated with the same material.

Notwithstanding these occasional discrepancies, it appears that isolation in mice alone is a reasonably dependable procedure, especially in human suspect cases.

When the rhesus monkey is used, there may be the rare instance in which no circulating virus is demonstrated although the animal may have been infected with or without a resultant temperature rise. Under such circumstances, blood serum taken a month or so after inoculation may be tested for specific antibodies in comparison with the pre-experimental serum. The presence of specific antibodies in the second specimen when these were absent from the first is indicative of a yellow fever infection of the animal in the interval between the examination of the two specimens. If all other possible sources of infection in the laboratory during this time can be eliminated, it may be concluded that virus was present in the original material.

From what has been said, it is clear that the origin of a virus recovered can be ascribed to a particular inoculum only to the extent that all other sources of virus have been eliminated. There is an uncanny tendency for unsuspected technical errors to occur at critical moments in an experiment. It cannot be overemphasized that the only means of ensuring the validity of a virus isolation is to have in the laboratory no virus source other than the inoculum under study

B. DEMONSTRATION OF SPECIFIC LESIONS

1. *Human cases.* In fatal cases of suspected yellow fever, autopsy may establish the diagnosis without delay. Grossly, there is evidence

pre- and postexperimental sera be tested in the same protection test run and that the criteria of interpretation be based on the behavior of the particular species toward virus under controlled conditions

II SEROLOGIC AND IMMUNOLOGIC PROCEDURES FOR DIAGNOSIS OF DISEASE

A INTRAPERITONEAL PROTECTION TEST

The intraperitoneal protection test involves the inoculation of a mixture of neurotropic yellow fever virus and the serum to be tested into the peritoneal cavities of a group of mice. When adult mice are used, starch solution is also inoculated intracerebrally to produce a localizing lesion. All methods in current use employ the French neurotropic strain of virus fixed for mice The technics of Sawyer and Lloyd,[1] Smithburn,[2] and Whitman[3] are presented below

1 *Adult mouse test (Sawyer and Lloyd*[1]*).*

a. "Specimens to Be Examined At least 6 cc of the serum to be tested are needed The test requires 3 cc The remainder is used in case the results of the first test are doubtful or the control tests prove unsatisfactory.

"The Mice Healthy young adult white mice of about 20 gm. weight are preferred, but moderate differences in age do not seem to affect the results of the tests A pure strain of mice, tested and found highly susceptible and bred on the premises, would give the best results Satisfactory work may be done with susceptible mice purchased from dealers, but each supply from a new source should be tested for susceptibility and there should be an understanding with the dealer that he will not change the source of his mice without notifying the laboratory During the test mice may be fed exclusively on bread soaked in milk, given once each day, and they will then require no water During the experiments the mice are kept in glass battery jars with wire mesh covers, six mice in each jar. Wood shavings are placed in the jars, and the mice are moved to fresh jars 1 week after inoculation

b "Preparation of the Virus An approximate time is set in advance for the tests, preferably 2 or 3 consecutive days in the middle of the week Five days before each testing date, a sufficient number of healthy mice are inoculated intracerebrally with yellow fever virus in mouse brain tissue The mice are inspected each morning. The definitely sick animals are killed with chloroform and are pinned out, back

point (50 per cent survival ratio) in a dilution of as high as 1:1,000 The absolute magnitude of the titers will depend on many factors in the conduct of the test, such as the amount of virus used and the contact time, hence the importance of including both the acute and convalescent phase sera in the same protection test.

If the first specimen has been obtained later in the course of the disease, it will have antibody, but the end point will be low; thus only a comparison of the end points will show whether there has been a rise in antibody content during the interval.

If the suspect disease is not yellow fever and the patient has not been previously immunized against yellow fever, neither the acute phase nor the convalescent phase serum will give protection against yellow fever virus, but if the patient has been previously immunized either by vaccination or by having had yellow fever, both sera will be protective and will give the same titer

D. APPLICATION OF THESE METHODS TO ANIMAL STUDIES

The three methods of diagnosing yellow fever which have been discussed above in their human application may be applied to studies in animals The most satisfactory is the immediate demonstration of virus by the inoculation of the infected animal's blood It should be remembered that the sera of many species of animals are toxic to mice when inoculated intracerebrally, but the effect disappears on dilution, and a preliminary test will suffice to show the degree of dilution necessary Dilution may be made with physiologic saline Attention to this factor may be necessary in protection tests

Histopathology tends to be less useful in animal studies than in human cases Many monkeys exhibit lesions similar to those occurring in human patients, but these animals are largely of the species in which the disease tends to cause high fatality. Most of the species important in the maintenance of jungle yellow fever have the disease in a mild form and show no characteristic lesions in the liver. In fatal cases, lesions may be present, but the histologic criteria of the human disease cannot be applied It is essential to study the manifestations of the disease in each species by itself in order to develop diagnostic criteria

The same comment applies to the interpretation of protection test results Many animal and bird species possess nonspecific neutralizing capacity for yellow fever virus upon which specific antibody behavior may be superimposed It is of even greater importance, therefore, that

and two should receive a known immune serum (human or monkey). To conserve the immune serum, it may be diluted to 10 per cent if of sufficiently high titer. The fifth control should be given the virus mixture intracerebrally. It shows whether the mice are susceptible and the virus potent. Intracerebral inoculation brings the animals down 1 day sooner than intraperitoneal inoculation with simultaneous cerebral injury.

f. "Inoculation of the Mice. An assistant anesthetizes the mice by placing several in a battery jar, on the bottom of which is a layer of cotton moistened with ether. The mice are taken from the jar when they become unconscious and are laid on the table near the operator. The operator, wearing rubber gloves, takes a mouse, lays it back up on a towel on the table, parts the hair of the head with a swab wet with 70 per cent alcohol and thrusts the fine needle of the tuberculin syringe through the thin skull and into the center of the brain He injects 0 03 cc of the starch solution. The mouse is then picked up by the skin of the neck, and its tail is held by the third finger of the same hand With one of the larger syringes an intraperitoneal injection of 0 6 cc. of the virus-serum mixture is given. The mouse is then dropped into a numbered jar One operator with an assistant can test up to 25 sera on each of 3 days of the week if he has an abundance of equipment ready in advance The actual injections require approximately 3 minutes for each mouse group

g. "Inspection and Recording The mice are inspected every morning for 14 days, and a record is made of those that are sick or dead. Our printed record form on cards measuring 77 by 128 mm is shown in Fig. 1. The first mouse to become sick or die becomes Mouse 1 of the group, and the second, No 2, and so forth

h "Interpretation of the Results The result of the protection test is recorded as the ratio of the number of mice surviving on the 10th day after inoculation to those that were alive and well on the 4th day. Deaths before the 5th day are in all probability not due to the yellow fever virus From the record cards the results are classified as 'protection,' 'no protection,' 'inconclusive,' or 'unsatisfactory,' in accordance with the requirements of the guide (table, p 276). Ordinarily the deferring of death beyond the 10th day shows a considerable protective action of the serum and would have almost the same significance as a survival If the amount of serum will permit, the tests of the specimens in the 'inconclusive' and 'unsatisfactory' groups are repeated,

down. A rapid and simple necropsy is performed to find out if there are gross lesions of any disease other than yellow fever and to drain the blood from the brain. The only gross lesion probably due to yellow fever, which has been observed, is hemorrhage into the stomach and intestines, and this is absent in most cases. The mouse is then turned over and pinned out. The skin is slit with scissors from the nose to the middle of the back and the flaps are laid back. An assistant sears the top of the skull with a red-hot soldering iron. The operator removes the bony covering over the brain with sterile sharp-pointed scissors and then spoons out the brain with a rigid, narrow spatula of nickel. The brain is placed in a small, weighed petri dish. The other brains secured are placed in the same dish and the total weight is ascertained. The brains are then finely ground up in a porcelain mortar with enough isotonic salt solution, or preferably salt solution containing 10 per cent of normal serum, to make a 10 per cent suspension.

c. "Preparation of the Serum-Virus Mixtures. In advance of the tests, 3 cc. of each specimen of serum or diluted serum and of each of several sera for the controls is placed in a small test-tube. If less than 3 cc. of serum is available for a test, a 50 per cent solution of the serum may be tested if the dilution is stated in the report. If less than 1.5 cc. is received, we discard the specimen unless its unusual importance justifies testing it in higher dilutions. To each tube is added 1.5 cc. of the virus suspension. The contents of each tube are mixed and drawn up into a 5 cc. graduated glass syringe with the number of the specimen or that of the mouse group to be inoculated written on it with a wax pencil.

d. "The Starch Solution. The starch solution for intracerebral injection is prepared in advance by adding 2 per cent of corn-starch, such as is sold for food, to the 0.9 per cent sodium chloride solution and heating in a flask in a bath of boiling water. The solution is then placed in small wide-mouthed Erlenmeyer flasks, autoclaved, and stored ready for use. In the morning of the day of the tests the starch solution is drawn up into tuberculin syringes of 0.25 cc. capacity, fitted with hypodermic needles 0.42 mm in diameter (Stubs gauge No 27) and 10 mm. long. The number of syringes filled is the same as the number of mouse groups to be inoculated.

e. "Controls With each set of tests there should be five control groups of six mice each Two groups should receive 0.4 cc. of normal serum (human or monkey) in place of the unknown serum of the test,

517 Mouse Group — *1137* Serum — *F-142* Virus — Control for

	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
1												†				
2															ǂ	
3															ǂ	
4															ǂ	
5															ǂ	
6															ǂ	

See card 511

529 Mouse Group — *Immune human* Serum — *F-142* Virus — Control for *511-528* — Control Groups — *(5/5)* Result

	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
1		†														
2												†				
3												†				
4															‡	
5															ǂ	
6															‡	

Source of Virus *See card 511*
Inoculum Intracerebral-Starch 2%, .005 cc.
Intraperitoneal -Virus 20%, .02 cc
Serum 100%, 0.4 cc.
Mouse Strain. *K*

533 Mouse Group — *Normal monkey* Serum — *F-142* Virus — Control for *511-528* — Control Groups — *(0/6)* Result

	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	May 26, 1931
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Date
1						S	†									
2							S	†								
3							S	M	†							
4							S	M	†							
5								S	†							
6								S	M	†						

Source of Virus *See card 511*
Inoculum Intracerebral-Starch 2%, .005 cc.
Intraperitoneal -Virus 20%, 0.2 cc
Serum 100%, 0.4 cc.
Mouse Strain. *K*

Key to symbols used in records

- † = Died
- ‡ = Killed when sick
- ǂ = Killed when well
- S = Sick
- M = Moribund
- F-142 = French strain of virus, 142nd passage in mice
- 6/6 (1/11) = 6 of 6 mice were protected in test; 1 of 11 survived in normal serum controls
- + = Serum protected
- ± = Result inconclusive
- − = Serum did not protect
- U = Test unsatisfactory

Heavy vertical lines bound time zone within which deaths are considered significant

Fig. 1. Part of record of test of a serum by the intraperitoneal protection test in mice, to determine protective power against yellow fever. Upper card contains record of test; other two show that of half the immune and normal serum controls.

and if definitely positive or negative results are then obtained they are accepted, but the protection ratios for both tests are shown in the tabular reports to the senders of the sera. The 'unsatisfactory' results include those in which the controls are unsatisfactory and those in which fewer than 4 mice are alive and well 4 days after inoculation "

GUIDE FOR INTERPRETATION OF THE 'INTRAPERITONEAL PROTECTION TEST' IN MICE

No of mice living and well 4 days after inoculation	No of deaths or survivals allowed among these mice 5 to 10 days after inoculation if the report is to be 'protection' or 'no protection' respectively *	
	Protection (+)	No protection or negative (—)
	No of deaths allowed	No of survivals allowed
1, 2, 3	Result 'unsatisfactory' (U) in any case.	
4	0	0
5, 6	1	1
7, 8, 9	2	2
10, 11, 12	3	3

* If there is neither 'protection' nor 'no protection' but the controls are satisfactory, the result is classed as "inconclusive" (±) If the results with the normal serum and known immune serum controls are not 'no protection' and 'protection' respectively, in conformity with the standards of the table, all the tests to which the controls relate are classed as 'unsatisfactory' (U) If possible, all 'inconclusive' and 'unsatisfactory' tests are repeated

2. *Adult mouse test of Smithburn* [4] At the Yellow Fever Research Institute, Entebbe, Uganda, the routine test is a somewhat modified form of the method of Sawyer and Lloyd described above. In the preparation of the virus, the infected mouse brains are ground in 10 per cent normal human serum in isotonic saline instead of saline alone as has become customary in many laboratories using the intraperitoneal test This permits a 1 per cent virus suspension to be employed and results in considerably greater sensitivity with no loss in specificity

The intraperitoneal inoculation of the serum-virus mixture and the intracerebral injection of starch solution are given almost simultaneously so that there is a constant relation between the times of the two inoculations. This is an important factor, and it will be noted above that Sawyer and Lloyd recommended this procedure originally. Nevertheless, it has been the general practice to inoculate the starch solu-

tion from 1 to 2 hours before doing the test, introducing thereby an additional source of variation and reducing the sensitivity of the test.

All other features of the test are the same as described for the standard intraperitoneal procedure.

3. *Young Mouse Test* (*Whitman*[3]) This procedure is being employed routinely by the United States Public Health Service in its laboratory at Hamilton, Montana. The technic is essentially the same as described by Whitman in his original publication of the method[3] In addition to its greater sensitivity, the test has the further advantage of employing less serum than the procedure of Sawyer and Lloyd. Amounts as small as 1 ml of serum may be tested satisfactorily.

The procedure being followed at Hamilton[4] is at follows:

a "Test virus. The French strain of neurotropic virus (400th to 500th passage in mice) is used. A suitable number of adult white Swiss mice approximately 50-60 days old are each injected intracerebrally with 0 03 cc of a 6 per cent suspension in physiological saline of freshly prepared mouse-brain virus Three days later all the living mice are killed with chloroform and the brains are removed with as little contamination as possible. Considering each brain to weigh 0 4 gram, a 20 per cent mouse-brain suspension in 10 per cent nonimmune serum-saline is prepared. This is done by using a sterilized enamel bowl (approximately 8 inches by 4 inches deep) and sash brush with bristles about 2 inches long. The shearing action by working the brush up and down and to the sides for several minutes will grind the brains much finer than a mortar and pestle. The diluent is next thoroughly mixed in, adding it slowly at first; then the virus suspension is centrifuged at 3,500 r p m. for 30 minutes The supernate is next transferred to a flask for distribution Cultures are made after centrifugation

b. "Serum-virus mixture The optimal virus dosage for each mouse is approximately 8 m l d (based on the 50 per cent end-point method of Reed and Muench). Experience has shown that 0 02 to 0 04 cc of the 20 per cent virus suspension described above gives close to this desired dosage

"The volume of serum for each mouse to be mixed with the virus is routinely 0 11 cc, although this may be varied at will from 0 04 cc to 0 36 cc, depending on the amount of serum available and the sensitivity of the test desired The serum virus mixture is made up on a basis of ten times volume received per mouse in order to allow for injection of 6 mice, possible injection of a 7th, and loss in manipulation

animals. The intracerebral technic of Theiler employs a standardized desiccated virus preparation so that a predetermined dosage can be given and the sensitivity thus regulated within considerable limits. With highly susceptible mice, a very sensitive test is obtained with a virus dose of the order of 25 m l d. (50 per cent mortality doses) whereas one of the order of 500 m l d will give results comparable to those of the standard intraperitoneal test of Sawyer and Lloyd. The serum required is 0.3 ml.

a. Procedure. The procedure given here is that of Theiler modified in minor respects by Bugher [6] and employed at Bogotá, Colombia, and the Yellow Fever Research Institute, Lagos, Nigeria. The test is also employed in a similar form but differing in minor features in the laboratories of the Yellow Fever Service, Rio de Janeiro, and in those of The Rockefeller Foundation, New York City. The differences are chiefly in the matter of contact time and temperature and the strain of mice used The procedure is as follows.

"Into a sterile Wassermann tube, 0.3 cc of the serum to be tested is pipetted Where the test is to be deferred, the cotton plug is cut off, flamed and depressed within the tube, and the mouth of the tube is closed by a previously sterilized cork or rubber stopper This prevents evaporation and with reasonable care there is no difficulty with the growth of molds beneath the stopper. The sera are grouped according to considerations detailed in a following section, and a sufficient number are selected to compose a 'run' The standard run is of 60 sera, exclusive of controls It is probably advisable to limit runs to a size permitting injection in not more than one hour

"In addition to the sera to be tested, the controls are established. One of these is made up of five tubes of normal human or monkey serum, or normal serum of the particular species of animal if but one species is being studied over a period of time The second control is a known immune serum which is preferably entered in the test as a titration, that is, serial dilutions are prepared using the standard diluent and 0 3 cc. amounts of each of the dilutions entered in the test Dilution ratios of 4 or 5 should be used and two dilutions above and two below the anticipated end point entered in the test. This necessitates, of course, preliminary standardization of the immune serum

"When all sera have been prepared for the addition of virus, they are listed in order on a work sheet and opposite each serum are entered the numbers of the mouse groups which are to receive the respective

"In advance of preparing the virus, the sera to be tested are measured into tubes bearing the respective serum serial numbers. Then the virus is carefully added to the side of each tube at a point about ½ inch above the serum. An assistant then carefully agitates each tube so that there is a complete mixing of the serum and the virus and no isolated drops of either serum or virus are left on the side of the tube.

c. "Controls. Each protection test run has 2 immune serum controls from different donors, 2 nonimmune serum controls from different donors and 1 virus titration control. The virus is titrated in 4-fold dilutions (1:1 through 1:256) using 12 mice for each dilution. At times a standard immune serum pool is titrated as a control in 4-fold dilutions, using a range sufficient to cover the end point.

d. "Inoculation of mice. Groups of 6 white Swiss mice 19 to 20 days old and weighing 9 to 10 grams each are injected intraperitoneally with each serum virus mixture as soon as all the tubes have been prepared and in the order in which the virus was added to the test specimens. A three-man team gives the best results—one to fill syringes, a second to anaesthetize the mice, and the third to inoculate the mice and carefully check all the serum and mouse group numbers with the work list.

e. "Interpretation of results. Assuming that the controls are satisfactory, interpretation of the results are given in the following table from Whitman's paper.

INTERPRETATION OF RESULTS OF INTRAPERITONEAL PROTECTION TESTS BASED ON SAWYER AND LLOYD [1]

Number of Mice Living on Fourth Day*	Number of Mice Living on the Tenth Day		
	Negative	Inconclusive	Positive
4	0	1, 2, 3	4
5	0, 1	2, 3	4, 5
6	0, 1, 2	3, 4	5, 6

* With less than four mice alive on the fourth day, the test is unsatisfactory."

B. INTRACEREBRAL PROTECTION TEST

1. *Method of Theiler.*[4] The intraperitoneal methods previously described all employ unstandardized virus suspensions of fresh mouse brain in saline or serum saline. The standard test also requires 3 ml. of serum, an amount which makes it ill adapted to studies in small

hemispheres will give a minimum of traumatic deaths" Ordinarily, 6 mice are inoculated with each mixture, but where finer discrimination is desired, 12 mice may be used

"As soon as the first lot of mixtures is injected, a second is removed from the refrigerator, and so on until all have been inoculated The purpose of this is to give as nearly the same time conditions to all mixtures as possible, the first tubes receiving somewhat less than the two hours and the last ones somewhat more. By placing the remaining tubes in the refrigerator, a further attempt is made to minimize the effect of the time lag Since the addition of the virus takes about 15 minutes and the injection requires one hour, the last tubes will have had slightly less than 2½ hours contact time, 45 minutes of which will have been spent at low temperature Ideally, the test should be injected in the time required to add the virus, but practically this is impossible.

"Smoothness in performance depends upon well-trained assistants. In addition to the operator, an anesthetist and one other assistant are needed. The anesthesia may be carried out in a glass battery jar and should proceed in such a way as to permit a continuous flow of mice in uniform anesthesia and at the rate at which they are being inoculated. Greater accuracy and speed are obtained if the mice have been previously placed in properly numbered boxes or jars.

"The details of the test are entered on the standard mouse cards and also in a separate protection test book The cards are used for the actual mouse record The duplication of these entries in the special book is worth the extra effort later when analysis of the results is carried out.

"The mice are observed each day at the same hour, and the sick and dead are recorded On the standard card, the first mouse to die becomes No 1 and so on The observations are continued for ten days, when all mice are discarded after the tenth day reading

b Materials and Controls "In the foregoing outline, it is evident that at every step are involved factors which can and do lead to variability in the results. It therefore becomes highly important to standardize each factor in order to minimize the over-all variability. The extent to which this is achieved determines the applicability of the test to the demonstration of small amounts of antibody

(1) "Mice As in the intraperitoneal test, it is highly important that the susceptibility of the mice be maximal and as uniform as pos-

serum mixtures. This sheet is the guide for the remainder of the procedure and greatly facilitates speed and accuracy in the final injection of the material. Since it is a guide for the immediate work and carries nothing that does not go into the permanent record, there is no necessity for keeping it.

"All being in readiness, two tubes of desiccated French neurotropic virus of an accurately standardized lot are rehydrated and a dilution is prepared which should have the calculated quantity of virus desired for the particular test, bearing in mind that, in making the mixtures, *the virus is diluted 1:2. To each tube 0.3 cc. of this virus dilution is* added, a 1 cc. pipette being employed and the tip of the pipette being *allowed to touch the interior surface of the tube about 1 cm above* the contained serum. On removing the pipette the tube is tipped and agitated *so that the serum washes every place touched by the virus and the* two are thoroughly mixed. This step is very important as the persistence *of a tiny droplet of virus on the side of the tube out of contact* with the serum may give an entirely different end result. This procedure *is carried out with each tube individually, but it can be done* very rapidly.

"Virus is added to all of the tubes, including those containing the *control sera, in exactly the same amount and manner* A portion of the virus is put aside in another tube for titration at the end of the test. This portion, about *2* cc., is allowed to stand with the test.

"*The entire rack of tubes is thoroughly shaken for about 30 seconds* as an added means of insuring complete mixing, and it is then allowed *to remain for the desired contact time without further attention* ,The contact time and temperature used are two hours at 37° C.

"With the nominal contact time of two hours, injection of the first mixture is begun 1¾ hours after *adding the virus. Ten or twelve tubes are taken from the rack and the remainder are placed in the refrigerator* Under uniform ether anesthesia the injections are made with a ½ cc tuberculin *syringe equipped with a No 27 gauge,* ½ inch needle. As *many sterile syringes should be ready as there are serum mixtures to* inject One finds a relatively soft area in the skull of the mouse in the *parietal region, through which the needle may be pushed very easily* A penetration *of 2 or 3 mm into the brain substance is sufficient* Deeper injection is likely to kill the mouse from pressure about the midbrain and brain stem, which is also true of inoculations carried out more posteriorly *Inoculation into either the right or left cerebral*

through a Seitz pad and dispensed in 5.0 cc. ampoules for use. Diluent is then made up as needed with sterile saline, no further filtration being necessary.

(4) "Volume of serum used The amount of serum used in this test is the least amount that permits the injection of 12 mice. If less than 0.3 cc. of serum is available, it may be augmented in volume by the addition of standard diluent to equal 0 3 cc. In any case, note should be made that the test was not carried out on the whole serum.

(5) "Contact time. The importance of the *in vitro* contact time has not been sufficiently emphasized in discussions of protection tests. In the intracerebral test, satisfactory and fixed contact time is fully as necessary as standardized virus.

(6) "Controls. The controls used in this test are three:

"Virus titration. A sample of the virus dilution used is allowed to stand with the test, and at the end tenfold dilutions are made (smaller ratios may be used if desired) and injected. Since the value of the virus is already approximately known, it is not necessary to cover a wide range in this titration.

"This virus titration thus represents the original virus plus whatever effect on it there may have been because of the diluent. With a well-stabilized virus preparation, an aberrant result in the titration, if no technical error has occurred, should lead to a re-examination of the diluent for virucidal activity. Ordinarily, this titration is the most valuable control, as it gives the data upon which to base the following test.

"Immune serum A pool of known immune serum, either human or monkey, is desirable for this purpose Where extensive work is being done with one animal species, it is convenient to establish an immune pool of that species. In any case, the pool should be large enough to permit its use over a prolonged period Greater value is secured from this control if it is set up as an antibody titration, serial dilutions of the immune serum being carried out to both sides of the end point

"Nonimmune serum Either as a separate control or included with the run itself, it is important to place a number of known nonimmune sera Routinely, 5 tubes of known nonimmune serum are inserted in each run."

c. Interpretation The results may be classified according to the schedule given under the adult mouse test of Sawyer and Lloyd This is the most commonly used procedure A somewhat finer measurement

sible from mouse to mouse. If, after a titration of French neurotropic virus in the mice, a dilution is prepared which contains 10 m l.d. as calculated by the method of Reed and Muench [7] and a new lot of mice is inoculated intracerebrally with 0.03 cc. they should, with very rare exceptions, all die. Almost all of the deaths will occur on the fifth to seventh days

"If the strain of mice used in any laboratory fails to give such uniform results on testing with virus, protection tests should not be started until a good strain is available. It is also wise to compare the susceptibility of a strain of apparently good mice with that of well-established colonies in other laboratories through the exchange of desiccated virus shipped in thermos bottles and packed in ice and carbon dioxide.

"We prefer to use mice from 42 to 50 days of age in order to keep this factor uniform, although there are no clear data to the effect that older mice are not equally satisfactory.

(2) "Virus. The virus is prepared as follows: Sufficient mouse brains infected with French neurotropic virus are removed to make 2 0 grams. This material is then ground in a sterile mortar, and small amounts of normal rhesus monkey serum or normal human serum are added until a total of 40 cc has been reached The emulsion is divided in 50 cc. centrifuge tubes and centrifuged at 2,500 r p.m. for 30 minutes; a shorter time at higher speed is better if the centrifuge permits. The supernates are collected and pooled. No gross particles should be visible if the supernatant portions are carefully taken off. Filtration is not necessary. The virus is placed in 0.5 cc. amounts in sterilized soft glass tubes 120 x 13 mm., or 5 cc. pyrex ampoules The dispensing is done with a 1 0 cc pipette to insure accuracy in volumetric measurement. The virus is then frozen and dried *in vacuo* by the technic described by Sawyer, Lloyd and Kitchen [8] and by Lloyd and Penna [9] or the more recent method of Bauer and Pickels [10]

(3) "Diluent The standard diluent used is 10 per cent normal rhesus monkey serum in 0 9 per cent saline solution The monkey serum is a pool made from sera of monkeys known to be negative in a sensitive protection test After making the pool, it is again tested as diluent by allowing the virus dilutions made in it to stand two hours at room temperature, titrations in mice being made at the beginning and end of the period. The loss of titratable virus should not exceed 20 per cent in this period

"As a precautionary measure, the pooled normal serum is filtered

mixture are spotted red while those from the 1:1,000,000 are colored blue Those six mice go into one jar. All inoculations are 0 03 cc. in amount and are made into the cerebrum without anaesthesia

b. "Interpretation: Since there are two mice for each dilution, it is evident that several patterns of result may occur in accordance with the following schedule:

Virus Dilution	Mortality			
1:500	+	+	+	—
	+	+	+	—
1:5,000	+	+	—	—
	+	+	—	—
1:50,000	+	—	—	—
	+	—	—	—
Interpretation	0	+	++	+++

+ Indicates death while — shows survival

Since the virus used kills regularly all the mice inoculated with the mixture made from the 1:500,000 virus (the final dilution is 1 1,000,000 after the diluent has been added) and is frequently irregular in the succeeding one which is twice this dilution, the dilution which kills all of the mice is said to have 1 m l d. so that in the test the three dilutions may give dosages of 600, 6,000 and 60,000 m l d per mouse

"The interpretation symbols have the following meaning therefor:

- 0 No protection
- \+ Feeble protection Serum neutralizes 600 m l d approximately
- ++ Strong protection Serum neutralizes 6,000 m l d approximately
- +++ Very strong protection Serum neutralizes 60,000 m l d approximately

As in the tests described previously, the mice are observed for 10 days and the deaths recorded"

Special attention has been paid at Dakar to the testing of icteric sera which are generally recognized as tending to give false neutralizations With such sera, the protein is separated and then reconstituted, eliminating the biliary products that tend to give false reactions False and true reactions can thus be distinguished

of the result may be obtained by the use of the "average survival time" as advocated by Bugher[8] wherein the time of death is considered in addition to the fact of death or survival. For diagnostic purposes, the use of the survival ratio is entirely sufficient. Those who may desire to use the average survival time criterion are referred to the published discussion of the method.

2. *Method of the Pasteur Institute, Dakar, French West Africa*[11] This technic differs in several respects from any other in common use today and, because it has been used in the large-scale vaccination program in French West Africa and in comparative studies of vaccine efficiency, it merits inclusion in this manual.

a. Procedure "The virus source is the desiccated whole brain substance of mice which have been infected with neurotropic yellow fever virus. Two such brains are ground in a mortar with 4 cc. of 10 per cent normal serum saline (human or monkey). This suspension is called a dilution of 1:5. From this, further dilutions in 10 per cent serum saline are made: 1:500, 1:5,000, 1:50,000 for the test itself and two additional dilutions 1:500,000 and 1:1,000,000 for the virus control.

"Three tubes are used for each serum and these are placed in a rack holding 25 columns of 3 tubes. All tubes of the front row receive 0.5 cc of the 1:50,000 virus, the middle row receives 0.5 cc of the 1:5,000 virus, the 1:500 dilution is dispensed in the same amount to the tubes of the back row

"The sera are then added. Beginning with the front tube (containing the most dilute virus) 0.5 cc. of the serum to be tested is added to each tube from front to back As the mixtures are made, each tube is shaken individually to insure that all virus is in contact with serum The rack is then allowed to stand one half hour at room temperature (25°–30° C).

"Two mice are inoculated intracerebrally with each dilution, using one syringe for the three mixtures beginning with the most dilute (front tube). The pairs of mice are spotted with yellow, blue, and red dyes in that order to distinguish the dilutions since all 6 mice are placed in one jar.

"After completing the inoculations of the test sera, the virus controls are completed by adding 0.5 cc. of diluent to 0.5 cc of each of the two final virus dilutions Without further incubation, these mixtures are inoculated into three mice each. Those from the 1:500,000

mixture are spotted red while those from the 1:1,000,000 are colored blue. Those six mice go into one jar. All inoculations are 0 03 cc. in amount and are made into the cerebrum without anaesthesia

b. "Interpretation· Since there are two mice for each dilution, it is evident that several patterns of result may occur in accordance with the following schedule.

Virus Dilution	Mortality			
1:500	+	+	+	—
	+	+	+	—
1:5,000	+	+	—	—
	+	+	—	—
1:50,000	+	—	—	—
	+	—	—	—
Interpretation	0	+	++	+++

+ Indicates death while — shows survival

Since the virus used kills regularly all the mice inoculated with the mixture made from the 1 500,000 virus (the final dilution is 1 1,000,000 after the diluent has been added) and is frequently irregular in the succeeding one which is twice this dilution, the dilution which kills all of the mice is said to have 1 m l d so that in the test the three dilutions may give dosages of 600, 6,000 and 60,000 m l d per mouse

"The interpretation symbols have the following meaning therefor:

- 0 No protection
- \+ Feeble protection Serum neutralizes 600 m l d approximately
- ++ Strong protection Serum neutralizes 6,000 m l d approximately
- +++ Very strong protection Serum neutralizes 60,000 m l d approximately

As in the tests described previously, the mice are observed for 10 days and the deaths recorded"

Special attention has been paid at Dakar to the testing of icteric sera which are generally recognized as tending to give false neutralizations With such sera, the protein is separated and then reconstituted, eliminating the biliary products that tend to give false reactions False and true reactions can thus be distinguished

of the result may be obtained by the use of the "average survival time" as advocated by Bugher[6] wherein the time of death is considered in addition to the fact of death or survival. For diagnostic purposes, the use of the survival ratio is entirely sufficient. Those who may desire to use the average survival time criterion are referred to the published discussion of the method.

2. *Method of the Pasteur Institute, Dakar, French West Africa.*[11] This technic differs in several respects from any other in common use today and, because it has been used in the large-scale vaccination program in French West Africa and in comparative studies of vaccine efficiency, it merits inclusion in this manual.

a. Procedure "The virus source is the desiccated whole brain substance of mice which have been infected with neurotropic yellow fever virus. Two such brains are ground in a mortar with 4 cc. of 10 per cent normal serum saline (human or monkey). This suspension is called a dilution of 1 5 From this, further dilutions in 10 per cent serum saline are made 1:500, 1:5,000, 1:50,000 for the test itself and two additional dilutions 1:500,000 and 1:1,000,000 for the virus control

"Three tubes are used for each serum and these are placed in a rack holding 25 columns of 3 tubes. All tubes of the front row receive 0 5 cc of the 1:50,000 virus, the middle row receives 0 5 cc. of the 1 5,000 virus; the 1:500 dilution is dispensed in the same amount to the tubes of the back row.

"The sera are then added. Beginning with the front tube (containing the most dilute virus) 0 5 cc. of the serum to be tested is added to each tube from front to back As the mixtures are made, each tube is shaken individually to insure that all virus is in contact with serum The rack is then allowed to stand one half hour at room temperature (25°–30° C).

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variable. Muench,[12] from statistical considerations applied to a large mass of data, has shown that the mortality distributions encountered are compatible with the expected frequencies in a complex sampling field made up of negative sera with a single mortality probability and positive sera whose corresponding mouse mortality probabilities themselves form a distribution, although the exact form of the distribution may not be known.

In the presence of such a situation, combined with variations inherent in the test itself, it is quite evident that the decisive differentiation between negative and weakly positive sera contain definite elements of arbitrariness. As in any quantitative procedure, as the variability rising from technical sources is reduced, more precise judgment may be exercised with respect to the phenomenon being measured. Also the working standard should be so established as to keep the significant errors at a minimum.

This last consideration has usually not been stressed sufficiently in regard to yellow fever protection test results For example, when human surveys are made to determine the regions in which yellow fever exists or has existed, it is highly important that the sera reported as positive should be true positives and indicate, beyond doubt, that the donors have had the infection. The matter of failing to demonstrate weak positives in such a program is distinctly a secondary one, so that it can hardly be said that the failure to differentiate weakly positive sera is a significant error, since specificity is the important thing in such sera as may be strongly positive. Therefore, for such purposes, a test with an abundance of virus may be desirable, with the sacrifice of maximum sensitivity.

If the object is to determine whether any antibodies exist, however, as in postvaccination studies or in animal experimentation, the failure to detect a weak positive may be as great an error as the report of a false positive. The standards in a case of this kind should be such as to make the totality of errors minimal.

It is to be emphasized, especially with animal sera, that the standards of interpretation must be established by the study of sera from known infected and noninfected animals Essentially, this involves an extensive study of the variability of results in animals known never to have been in contact with yellow fever virus but which may be presumed to reflect all other factors which may be operating in the region under consideration

C. COMMENT

The mouse protection tests described here are in use in various laboratories where yellow fever is being studied, and they form the basis for many publications which have appeared. It is important to realize that there are differences between them in such matters as sensitivity. The test being used at Dakar is by far the most severe, in that a large amount of virus must be neutralized in order that all of the mice inoculated with a mixture may survive. The method of Sawyer and Lloyd as usually performed also has a low order of sensitivity although when the original technic is followed, it is considerably more sensitive. The use of 1 per cent virus in 10 per cent normal serum saline with simultaneous starch injection and intraperitoneal inoculation gives a sensitivity of the same order as that of the intracerebral test of Theiler and of the young mouse test of Whitman The 1 per cent test, however, requires 3 ml. of serum as compared with 0 3 ml. needed for the intracerebral method It will be found that the sensitivities of all these methods will differ in various laboratories, partly from variations in technic but especially from differences in mice.

With the exception of the method of Whitman, none of those given requires mice of critical age. Very satisfactory tests may be made in suckling mice, but such methods have not been included since they presuppose the existence of a mouse colony and are applicable only in highly specialized circumstances.

Similarly, other serologic methods, such as complement fixation, have not been included. These tests have not the conclusive diagnostic value of the neutralization reactions and have not as yet the general applicability.

It should also be recognized that the schedules of interpretation which have been given really apply specifically to human sera. It will be found that they may be used with satisfaction for nearly all monkeys and apes as well, but their extension to orders other than the primates should be made with care.

At first thought, one is inclined to say that a properly established protection test is one in which all mice inoculated with serum mixtures without antibody content should die, while all those injected with antibody-containing mixtures should survive A brief experience with animal as well as with human sera following known infection with yellow fever or vaccination with living virus is sufficient to demonstrate that the virus-neutralizing capacities of such sera are quite

variable Muench,[12] from statistical considerations applied to a large mass of data, has shown that the mortality distributions encountered are compatible with the expected frequencies in a complex sampling field made up of negative sera with a single mortality probability and positive sera whose corresponding mouse mortality probabilities themselves form a distribution, although the exact form of the distribution may not be known.

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A further word of caution concerns the general technical background for these as well as all other tests involving virus neutralization. All glassware must be meticulously cleansed if one is to avoid introducing systematic errors resulting in false neutralizations. Residues of alkaline washing powders and soaps as well as of oxidizing cleaning agents may destroy the virus and give false reactions, particularly with the methods employing small amounts of serum where the wetted surface of the glassware is relatively large in comparison with the volume of the liquid. Where great certainty is desired concerning any one result, the test should be repeated, preferably with another bleeding of the same donor.

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DENGUE

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I. INTRODUCTION

DENGUE is an infectious, mosquito-transmitted disease, characterized, as a rule, by fever of 5 to 7 days' duration, by pain in various parts of the body which may be severe enough to lead to prostration, and by morbilliform or scarlatiniform rash, lymphadenopathy, and leukopenia In nature the only known mode of transmission is by mosquitoes of the genus *Aëdes* and the only species incriminated thus far are ***aegypti, albopictus,*** and *scutellaris.* Dengue is suspected, therefore, whenever a disease having some or all of the clinical manifestations just mentioned occurs at a time when these mosquitoes are present in appreciable numbers Until recently it was generally believed that very little immunity followed a single attack of the disease. It has been established, however, in the last few years that multiple immunologic types of the virus exist, and that immunity to homologous types can be of long duration. It is noteworthy, however, that infections caused by heterologous immunologic types, especially within a few months after the first attack, may produce illnesses that are mild, without rash, and associated with fever that may be only of 1 to 3 days' duration

The dengue virus is present in the blood in large amounts during the first 24 hours after onset of fever but, by inoculation of susceptible human beings, can be detected in diminishing concentration as long as the fever persists The virus as it is contained in human serum is readily filtrable, is not larger than 17 to 25 mμ, as determined by filtra-

A further word of caution concerns the general technical background for these as well as all other tests involving virus neutralization. All glassware must be meticulously cleansed if one is to avoid introducing systematic errors resulting in false neutralizations. Residues of alkaline washing powders and soaps as well as of oxidizing cleaning agents may destroy the virus and give false reactions, particularly with the methods employing small amounts of serum where the wetted surface of the glassware is relatively large in comparison with the volume of the liquid. Where great certainty is desired concerning any one result, the test should be repeated, preferably with another bleeding of the same donor.

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mice (a light brown strain originally bred at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine) should be used. If the "dba" mice are not obtainable, very young Swiss albino mice may be used. Each of one group of 10 mice receives intracerebrally 0 03 ml. of the undiluted serum, and each of another group of 10 mice the same volume of a 1:100 dilution in physiologic salt solution, which is done in an attempt to dilute an inhibitor if one should be present. The mice are observed for at least 5 to 6 weeks, and any mouse which develops motor weakness or paralysis or dies during that time should be used for passage. A 20 per cent suspension of its spinal cord and brain is inoculated intracerebrally into another group of 10 mice for passage. If some of the material is to be stored in dry ice, it should be suspended in undiluted rabbit serum which has been heated at 56° C. for 30 minutes Many passages may be needed before a strain of virus is obtained which will produce signs of nervous system involvement in all inoculated mice, and many more passages before it reaches an appreciable intracerebral titer. The final proof that a mouse-adapted strain obtained in this manner is a derivative of the original dengue virus depends on the demonstration that the mouse virus will produce complete immunity to the original human virus by tests in human beings Neutralization by a specific rabbit serum may be equally significant; on the other hand, neutralization by a human serum containing dengue antibodies would be significant only if that serum is known to be free of antibodies to other viruses, such as herpes, vaccinia, or yellow fever. Lack of neutralization by a serum known to contain the homologous dengue antibody, however, would indicate that the virus being passed in mice was picked up during passage and was not derived from the original human dengue virus.

III. SEROLOGIC PROCEDURES FOR DIAGNOSIS

Human beings recovering from experimental infection with dengue virus have been found to develop neutralizing antibodies for the homologous immunologic type of mouse-adapted virus. These antibodies have been detected as early as one week after onset (first day of defervescence) and as late as 2 years after a single attack, and may persist for longer periods The antibodies have also been detected in the sera of patients 2 years after a natural attack of the disease during epidemics in Hawaii and Japan The exact antigenic pattern of the different immunologic types of dengue is still in the process of

tion through gradocol membranes, and can be preserved for at least 3 years either in the frozen state with the aid of dry ice or in the dry state after proper lyophilization. The human virus produces only a low-grade febrile or inapparent infection in monkeys, and, as a rule, is practically without effect in the ordinary laboratory animals or embryonated eggs. However, several strains of the virus have now been adapted to mice by laborious procedures which are not suitable for routine diagnostic purposes. The mouse-adapted dengue virus is infectious only by the intracerebral route and may be differentiated from other known viruses by its limited host range in laboratory animals At this time, the mouse-adapted dengue virus is unique in being pathogenic for mice and not for cotton rats or hamsters; it also is not pathogenic for rabbits or guinea pigs, but it can give rise, after intracerebral injection in rhesus monkeys, to an inapparent or low-grade febrile infection, followed by the development of neutralizing antibodies, and under certain conditions to a poliomyelitis-like paralytic disease.

II. METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

No methods of virus isolation are suitable for diagnostic purposes. To obtain a strain of virus for research purposes, the serum from suspected cases, previously shown to be nonpathogenic for mice, guinea pigs, and rabbits, has been inoculated intracutaneously, subcutaneously, intramuscularly, or intravenously in susceptible human beings. A strain of dengue virus may be considered to have been recovered when the characteristic features of the disease are reproduced after an appropriate incubation period (usually 5 to 7 days) and further passage and transmission by *Aedes aegypti* mosquitoes can be established. Intracutaneous injection of the serum is especially useful because the dengue virus produces a local skin lesion after an interval of 3 to 5 days, whereas serum from cases of sandfly fever (pappataci or phlebotomus fever) does not. Neutralization tests with known dengue immune sera or cross-immunity tests with known strains of virus are helpful when positive, but of no significance when negative because of the existence of multiple immunologic types. When the serum from suspected cases needs to be transported over long distances, it may be packed in ordinary ice if dry ice is not available.

The following procedure is recommended for attempts to adapt dengue viruses to mice Whenever possible, 2- to 3-week-old, "dba"

hours Without this incubation, sera containing a moderate amount of antibody are negative, and those with a great deal of antibody show only slight neutralization The protection exerted by the interference phenomenon requires no preliminary incubation and thus differs from that effected by the neutralizing antibodies

6. After incubation, 0 03 ml. of each dilution is injected intracerebrally into each of 5 mice which are 3 to 4 weeks old. The mice are observed for at least 28 days after inoculation because at present the incubation period can be as long as 22 to 24 days, particularly when neutralization is only partial

7. The neutralization index is calculated from the LD_{50} titers of the mixtures as is done in the tests with other viruses. Specific neutralization indexes have always been more than 50 and not infrequently 1,000 or more; for this reason 50 was selected as the minimum significant index. It has been found that sera from patients experimentally infected with heterologous types of dengue virus usually yield partial neutralization in the form of prolonged incubation periods and indexes of 20 to 40. While this has been observed with sufficient regularity to be regarded as significant, it is not recommended as a basis for diagnosis of infection with a heterologous type of dengue

IV. REFERENCES

The material presented in this chapter is based for the most part on recent studies by Sabin and Sabin and Schlesinger, which are, except for a preliminary report, unpublished

Sabin, A B, and Schlesinger, R W Production of Immunity to Dengue with Virus Modified by Propagation in Mice *Science*, 101 640–642 (June 22) 1945

The work until 1943 is reviewed in·

Lumley, G F., and Taylor, F H Dengue Service Publication No 3, School of Public Health and Tropical Medicine (University of Sydney), Department of Health, Commonwealth of Australia

investigation, but it is known that the antibody response in human beings is sufficiently type-specific to limit the significance of results obtained with the single immunologic type of mouse-adapted dengue virus available for routine diagnostic work at this time. A positive result with a neutralization index of 50 to 100 or more can be interpreted as indicating past infection with dengue, most probably of the same type used in the test, a negative or equivocal result does not mean absence of dengue infection. As in other virus diseases, in order to prove by serologic methods that a given infection was caused by dengue virus, it is necessary to establish that the antibodies were either absent or present in low concentration during the acute phase of the illness and developed or increased in amount during convalescence. In dengue it has been observed that the serum taken from experimental cases on the first day of fever may have a protective effect against the mouse-adapted virus, which is due to the interference phenomenon and not to neutralizing antibodies. The effect of serum obtained later during the febrile phase has not been investigated as yet, but this phenomenon must be kept in mind in neutralization tests with dengue viruses.

A. NEUTRALIZATION TEST

The technic of the neutralization test developed by Sabin and Schlesinger is as follows

1 The patient's serum for the test is frozen in dry ice or lyophilized as soon as possible, because the antibody is unstable, heating at 56° C. for 30 minutes can destroy the antibody completely

2. The virus to be used in the test is stored in dry ice as a 20 per cent, centrifuged suspension of the brain and cord of infected mice in undiluted rabbit serum which has been heated at 56° C for 30 minutes. The intracerebral titer of this virus should be at least 1 1,000

3. Serial 10-fold dilutions of the original 20 per cent suspension are made in undiluted, heated rabbit serum to yield 1 50, 1 500, 1.5,000, *and further* dilutions of the virus, the actual number of dilutions that are prepared depends on the potency of the virus and should be sufficient to permit calculation of the LD_{50} titer.

4. The mixtures are prepared by adding 0 2 ml of each of the dilutions to 0 2 ml. portions of the undiluted serum that is being tested. The virus control mixtures are similarly prepared with either known negative undiluted human serum or normal rabbit serum

5. The mixtures must be incubated in a water bath at 37° for 2

hours Without this incubation, sera containing a moderate amount of antibody are negative, and those with a great deal of antibody show only slight neutralization. The protection exerted by the interference phenomenon requires no preliminary incubation and thus differs from that effected by the neutralizing antibodies.

6. After incubation, 0 03 ml. of each dilution is injected intracerebrally into each of 5 mice which are 3 to 4 weeks old The mice are observed for at least 28 days after inoculation because at present the incubation period can be as long as 22 to 24 days, particularly when neutralization is only partial.

7. The neutralization index is calculated from the LD_{50} titers of the mixtures as is done in the tests with other viruses. Specific neutralization indexes have always been more than 50 and not infrequently 1,000 or more, for this reason 50 was selected as the minimum significant index. It has been found that sera from patients experimentally infected with heterologous types of dengue virus usually yield partial neutralization in the form of prolonged incubation periods and indexes of 20 to 40 While this has been observed with sufficient regularity to be regarded as significant, it is not recommended as a basis for diagnosis of infection with a heterologous type of dengue

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PHLEBOTOMUS (PAPPATACI OR SANDFLY) FEVER

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I. INTRODUCTION

PHLEBOTOMUS (pappataci or sandfly) fever is an infectious, phlebotomus-transmitted disease of virus etiology characterized, as a rule, by fever of 2 to 4 days' duration, severe headache, pain in the eyes, generalized malaise, and leukopenia The disease is self-limited and without fatalities The only proved vector is *Phlebotomus papatasi*, and mosquitoes (*Aedes aegypti* and *Culex pipiens*) have been found incapable of transmitting the virus under optimum experimental conditions. Outbreaks of phlebotomus fever are suspected, therefore, whenever an illness having the characteristics mentioned above occurs, especially among immigrants, tourists, or foreign troops in countries harboring the vector and during the hot, dry season (April to October) when the vector is most likely to be prevalent.

The virus is present in the blood during the first 24 hours of the fever, but the maximum amount found in the serum thus far is only 1,000 human infective doses per ml. The virus has been preserved in dry ice or in the lyophilized state for approximately 4 years The disease is readily reproduced in susceptible human beings when the serum is injected intracutaneously or intravenously but only irregularly when it is injected subcutaneously or intramuscularly. Serum, infective for human beings, has been found to be without effect in a large variety of animals There is now no laboratory animal available for this virus and no satisfactory, confirmed evidence that the virus can be propa-

does not signify that the virus may not belong to the phlebotomus fever group. Failure of transmission by *Aedes aegypti* mosquitoes is important for differentiation from yellow fever and the dengue group of viruses When immunologic and insect transmission tests cannot be carried out readily, determination of size by filtration through gradocol membranes may help in the presumptive classification of the unknown virus.

III. SEROLOGIC PROCEDURES FOR DIAGNOSIS

The neutralization tests in human beings cannot be recommended even for unusual circumstances because of their irregularity, and no other procedures are available at this time.

IV. REFERENCES

The material presented in this chapter is based in large measure on unpublished studies of the author. For recent review, see:

Sabin, A B., Philip, C. B., and Paul, J R. Phlebotomus (Pappataci or Sandfly) Fever; a Disease of Military Importance; Summary of Existing Knowledge and Preliminary Report of Original Investigations. *J.A.M.A*, 125.603, July 1, 1944; 693, July 8, 1944

gated in embryonated eggs. The plaquelike lesions on the chorioallantoic membrane reported by some investigators have been found by the writer to be nonspecific and not associated with propagation of the virus. The virus in human serum has been found to pass 200 mμ gradocol membranes but not membranes with an average pore diameter of 100 mμ or less. The size of the virus has, therefore, been estimated as being not larger than 40 to 60 mμ although it may be smaller. A strain of virus recovered during an outbreak of the disease among American troops in Naples, Italy, has been found to be immunologically different from 2 strains of virus recovered the preceding year during outbreaks in Sicily and the Middle East It has been established in the last few years that immunity to infection with homologous strains of virus can be of long duration following a single experimental attack of the disease, the longest period tested thus far being 2 years However, tests for neutralizing antibody carried out in human volunteers have yielded irregular results, suggestive evidence of neutralization having been obtained only when small amounts of virus were used

II. METHODS OF ISOLATION AND IDENTIFICATION OF THE VIRUS

There are no methods of isolation which are suitable for routine diagnostic purposes To obtain a strain of virus for research purposes, blood is drawn from suspected cases, not later than 24 hours after onset, and the serum is stored on dry ice until the clinical course of the disease in the human donors is found to be compatible with a diagnosis of phlebotomus fever, and tests in mice, guinea pigs, and rabbits indicate the absence of an infectious agent pathogenic for these animals The serum should then be injected intracutaneously or intravenously in doses of 1 to 2 ml in human volunteers A strain of virus may be considered to have been recovered when a febrile illness of 1 to 4 days' duration (rarely longer) associated with leukopenia but without rash is reproduced after an incubation period usually of 3 to 7 days (may be 40 hours after intravenous injection), and further passage is possible Absolute identification of the transmissible, filtrable agent may not be possible without the demonstration that it can be transmitted by *Phlebotomus papatasi*, especially if the agent proves to be immunologically distinct from available proved strains of the virus Thus, cross-immunity tests in carefully selected human volunteers when positive can help to identify the unknown virus, but lack of cross-immunity

does not signify that the virus may not belong to the phlebotomus fever group. Failure of transmission by *Aedes aegypti* mosquitoes is important for differentiation from yellow fever and the dengue group of viruses. When immunologic and insect transmission tests cannot be carried out readily, determination of size by filtration through gradocol membranes may help in the presumptive classification of the unknown virus.

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RICKETTSIAL DISEASES

PART I

Isolation and Identification of Rickettsial Agents

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I. INTRODUCTION

THE rickettsial diseases are a group of illnesses that occur throughout the world. They are caused by the rickettsiae, tiny microorganisms midway between the bacteria and the filtrable viruses, requiring living cells for proliferation, staining poorly with ordinary bacteriologic stains, and for the most part transmitted by arthropods The microorganisms are named after Ricketts, who first saw and described them in cases of Rocky Mountain spotted fever in 1908. Subsequently, in 1910, Ricketts noted similar organisms in body lice infected with typhus fever.

The rickettsiae and the diseases they cause can be classified by several different schemes. By some the diseases are spoken of as the typhus fevers and are classified according to the transmitting vector, such as louse typhus, tick typhus, and mite typhus. This classification, however, proves to be inadequate when more than one rickettsia can be transmitted by the same vector or when applied to Q fever, which apparently can be contracted in the absence of known arthropod vector. A more satisfactory classification is one based upon the etiologic agent per se and its antigenic relationship to others in the group A working classification of the rickettsial diseases and their causative agents follows

1 Typhus Group
    a) Epidemic (*R prowazeki*)
    b) Murine (*R mooseri*)
2 Spotted Fever Group
    a) Rocky Mountain spotted fever (*R. rickettsi*)
    b) Boutonneuse fever (*R conori*)
    c) Rickettsialpox (*R. akari*)
3. Tsutsugamushi Disease or Scrub Typhus (*R. orientalis*)
4. Q Fever (*R. burneti*)

A. GENERAL CHARACTERISTICS OF RICKETTSIAL AGENTS

Rickettsia prowazeki, the cause of epidemic typhus, is the type species of the genus. Although this organism can be demonstrated in infected animal tissues and in the gut of infected lice, it is easily seen in preparations from the yolk sac of infected hen's eggs; here, under proper conditions, the rickettsiae grow well. Rickettsiae stained by Macchiavello's method are red whereas with Giemsa's technic they stain a pinkish purple The rickettsiae show considerable variation in

size, from almost invisible particles to rods that may reach more than 2 μ in length. Diplococcoid forms are frequently seen, particularly in preparations of *Rickettsia orientalis*.

The rickettsiae will not grow except in the presence of living cells *Rickettsia burneti* has been shown to pass the bacterial filters consistently [1] and under certain conditions so will *Rickettsia prowazeki*.[2] Most of the rickettsiae can be sedimented by centrifugation in the small angle centrifuges at 3,500 to 4,000 r.p m. A soluble antigen (probably protein) has been described as being released from *Rickettsia prowazeki, Rickettsia mooseri*, and *Rickettsia rickettsi*, upon exposure to diethyl ether.[2, 3] This soluble antigen is capable of eliciting all the immunologic responses that are obtained with formalin inactivated intact organisms. By means of the electron microscope, it appears that some of the rickettsiae (*Rickettsia prowazeki* and *Rickettsia mooseri*) are enveloped by a capsular substance which may be the source of the soluble antigen.[4]

Recently, a new member of this group, *Rickettsia akari*, has been added by Huebner of the National Institute of Health [5] This organism is the etiologic agent of a disease, given the name of Rickettsialpox, described in 1946 in New York City The disease is mild, with an initial lesion and an exanthem that has a tiny vesicle as the main differentiating characteristic from other rickettsial rashes The organism is a typical rickettsia, apparently transmitted to man by a mite from mice, it grows well in the yolk sacs of fertile eggs, is pathogenic for guinea pigs and mice, and is related to but not identical with *Rickettsia rickettsi*, the cause of Rocky Mountain spotted fever

II. ISOLATION AND IDENTIFICATION OF THE RICKETTSIAE

The laboratory diagnosis of the rickettsial diseases depends, in general, upon two forms of tests: (1) the isolation and identification of the causative agent, and (2) the demonstration of a rise in antibody in the patient.

A. PRECAUTIONS FOR WORKERS

Many of the procedures used in the laboratory diagnosis of the rickettsial diseases expose the personnel to a danger of accidental infection The risk may be modified, however, by the vaccination of personnel, particularly against Rocky Mountain spotted fever and epidemic and murine typhus The rickettsiae, apparently, are highly infectious,

and accumulated laboratory experience indicates the danger from airborne infection as well as from breaks in technic.

B. Sources of Material

1. *Human cases.* The etiologic agent in the rickettsial diseases circulates in the blood of man at least early in the disease. In the guinea pig, inoculated intraperitoneally with from 3 to 5 ml. of whole blood drawn early in the course of the disease, isolations are possible in 60 to 75 per cent of the cases of Rocky Mountain spotted fever and in epidemic and murine typhus. There is an incubation period of 2 to 10 days, depending upon the strain involved. For example, a strain of Rocky Mountain spotted fever, highly virulent for the guinea pig, has an incubation period of 2 days; with a mild strain it is prolonged to 5 or 7 days. After the incubation period, the guinea pig's temperature becomes elevated for a variable number of days, again depending upon the strain involved. Temperatures above 39.6° C. are usually considered fever in the guinea pig.

2. *Arthropods.* The arthropods that transmit the rickettsial diseases frequently serve as sources for the isolation of strains of rickettsiae These are the ticks (*Dermacentor andersoni, Dermacentor variabilis, Amblyomma americanum,* and others) in spotted fever, the human body louse (*Pediculus humanus*) in epidemic typhus, the fleas (*Xenopsylla cheopis*) in murine typhus, the mites (*Trombicula akamushi, Trombicula deliensis*) in tsutsugamushi disease, and the mite (*Allodermanyssus sanguineus*) in rickettsialpox.

3. *Reservoirs.* In certain laboratory studies of strains of the rickettsiae, it may be necessary or advantageous to attempt isolations from some of the natural reservoirs of the disease Some of these are the wild rodents in Rocky Mountain spotted fever, rats and mice in murine typhus, wild rodents in tsutsugamushi, house mice in rickettsialpox.

C. Collection of Material

1. *Human cases.* Since the blood of human cases is infectious, particularly during the febrile illness, sterile blood is collected by venipuncture either into ampules or into a sterile syringe. This blood can be injected as whole blood immediately into the peritoneal cavity of guinea pigs

2. *Arthropods* The arthropods may be collected in a variety of ways. Ticks can be picked from animals in the field, such as dogs,

cattle, or small rodents, or they may be collected by flagging This is accomplished by using a small flag of white outing flannel with a suitable wooden handle for dragging the low underbrush along the side of roads and trails. The ticks can be picked from the outing flannel with small forceps and dropped into vials for later use Fleas can usually be collected best by the combing of trapped rats or mice. The animals are anesthetized with ether and then a fine comb is run through their hair. It may be necessary to dampen the fur with a disinfectant to facilitate the collection of the fleas. Mites are very tiny and difficult to collect. A suction apparatus, somewhat similar to that used in the collection of mosquitoes, is frequently necessary, and the collection can be made from boot tops and pant legs of the collector after passage through an area where mites are present

3. *Reservoirs.* The reservoirs of importance in the rickettsial diseases are mainly the wild rodents Animals for study should be collected alive if at all possible, several satisfactory types of traps have been devised for live trapping of rats and mice

D. Preparation of Materials for Inoculation

1. *Human cases* The sterile blood collected from the human case, if early, can be used as whole blood; if late (the middle or last of the second week of febrile illness) it is best to allow the blood to clot so that the antibody-containing serum can be separated from the clot. The clot is then macerated in a hand mortar with sterile sand or alundum, diluted to the original volume with sterile saline solution, and 3 to 5 ml inoculated into the peritoneal cavity of guinea pigs

2 *Arthropods* The arthropods that have been collected should be washed in ether to remove the surface contaminants The ether should then be allowed to evaporate at room temperature, after which the arthropods should be washed once or twice in sterile saline solution. They may then be macerated in a hand mortar with sterile sand or alundum, diluted with approximately 3 ml of saline solution for each arthropod, or 10 to 15 ml of sterile saline solution for lots of 50. The suspension is allowed to settle, the supernatant is drawn into a sterile syringe, and then 3 to 5 ml are inoculated intraperitoneally into the guinea pigs

3. *Animals* If the animals that have been collected are to be used for isolation of the agent of Rocky Mountain spotted fever, pieces of the liver and spleen are removed aseptically and ground in a hand mortar with sterile sand or alundum Sufficient sterile saline is added

to make approximately a 10 per cent suspension, which is then allowed to settle, and 3 to 5 ml. of the suspension are inoculated intraperitoneally into guinea pigs. For the isolation of the agents of murine or scrub typhus from rats and mice, the brain is removed aseptically, is ground in a sterile mortar with a suitable abrasive, is made into a 10 per cent suspension with sterile saline, and 3 to 5 ml. of the supernatant are inoculated intraperitoneally into guinea pigs.

E. SELECTION OF ANIMALS

A variety of ordinary laboratory animals is susceptible to infection with the rickettsiae. The guinea pig is probably the most universally used animal for investigations. It is susceptible to intraperitoneal infections with all the rickettsiae. A febrile illness following an incubation period of usually 3 to 6 days is observed, and, with *Rickettsia mooseri* and *R. rickettsi*, typical scrotal reaction can be seen.

White mice are satisfactory for investigations involving *Rickettsia orientalis* Death occurs usually in 5 to 20 days following intraperitoneal inoculations, depending upon the dilution of the inoculum. Mice can also be used for investigations of Q fever, and even though death does not occur, some 7 to 10 days following inoculation they have enlarged spleens in which rickettsiae can be microscopically demonstrated

Monkeys are quite susceptible to infections with *Rickettsia rickettsi* and have also been used in studies of the other rickettsial agents

A wide variety of rodents, both domesticated and wild, have been shown to be useful for various investigative purposes in studies of the rickettsiae.[6, 7, 8]

F. REACTIONS IN ANIMALS

1. *Scrotal reactions in guinea pigs* In several of these infections, male guinea pigs develop a scrotal reaction which may be of considerable importance in classifying the strain. In Rocky Mountain spotted fever, the scrotal reaction usually develops after the guinea pig has had fever for 4 to 7 days. Some animals may die before the scrotal lesions develop. The reaction is first evidenced by a very slight diffuse erythema, and at this stage the scrotum becomes stretched and assumes a shiny appearance. The following day a rash is seen over the scrotum which may extend upward onto the groin. These macules become darker very rapidly so that within 24 hours the skin of the scrotum appears purpuric where the dark purplish macules have

coalesced. The superficial layers of the skin slough, leaving ulcers which may have more or less bizarre shapes. Healing begins and is complete in about 5 days, leaving permanent scars on the scrotum The mild strains of spotted fever usually do not produce these lesions

A characteristic scrotal reaction is produced in male guinea pigs by a murine strain of typhus fever. This reaction is entirely different from that of Rocky Mountain spotted fever. In murine typhus, the reaction is usually seen on the first day of fever. Here the deeper structures are involved rather than the superficial skin. The tunica vaginalis becomes edematous with marked injection. The scrotum appears swollen, and it is no longer possible to replace the testicles through the external ring into the abdominal cavity Other than that the skin of the scrotum is tightly stretched and reddened; there are no superficial lesions After 2 or 3 days, the reaction subsides, with no permanent evidence of the previous pathologic changes There are no consistent characteristic scrotal reactions with an epidemic strain of typhus fever, Q fever, or tsutsugamushi disease

2. *White mice* White mice are easily infected by intraperitoneal injections of blood from the patient with tsutsugamushi disease. If infected, their fur becomes ruffled some 6 to 8 days later, and they appear ill; by the 10th to 12th day their abdomens become distended with a clear viscous fluid, and death occurs usually from the 12th to the 15th day. Smears are made by lightly scraping the peritoneal cavity and when stained with methylene blue, Giemsa, or Macchiavello, reveal rickettsiae in the cells A rickettsial pneumonia can be produced in white mice inoculated intranasally with *Rickettsia prowazeki*,[9] *R. mooseri*,[10] and *R orientalis*[11] and the lungs can be used as a source for preparing rickettsial suspensions.

G METHODS OF PASSAGE

During the first 3 or 4 days of fever, the different strains can be passed to other guinea pigs by using 1 or 2 ml of whole blood or a suspension of some of the organs, such as brain, liver, spleen, or the washings of the tunica vaginalis Recovered animals are solidly immune to subsequent infection with a homologous strain, partially immune to closely related strains, but not to the other rickettsiae. Murine and epidemic typhus, however, exhibit almost complete reciprocal cross-immunity One of the standard methods of identifying an unknown strain is to study the cross-immunity reactions with known strains

to make approximately a 10 per cent suspension, which is then allowed to settle, and 3 to 5 ml. of the suspension are inoculated intraperitoneally into guinea pigs. For the isolation of the agents of murine or scrub typhus from rats and mice, the brain is removed aseptically, is ground in a sterile mortar with a suitable abrasive, is made into a 10 per cent suspension with sterile saline, and 3 to 5 ml. of the supernatant are inoculated intraperitoneally into guinea pigs.

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70 per cent alcohol with 1 to 2 per cent "Roccal" (Winthrop), and the alcohol is burned while the shell is still wet. The air sac is broken open with a sharp blow, this part of the shell is removed piece by piece with sterile forceps, and then the air sac membrane is removed The entire contents of the egg are then poured into a sterile petri dish and the yolk-sac tissue freed and transferred to another petri dish with sterile forceps. A small piece of tissue is teased from the membrane, and yolk fluid is removed by wiping on a glass surface (petri dish or slide), then the bit of tissue is macerated over the slide The tissue impression smear is then stained by Macchiavello's method or by Giemsa's method. The latter technic is well known; details of Macchiavello's method are as follows: A 0 25 per cent solution of basic fuchsin is made either in a phosphate solution, buffered at 7.4 pH or in distilled water brought to 7.2 to 7.4 pH with sodium hydrate or sodium carbohydrate Preparations are made by smearing a bit of tissue on the slide, drying gently by heat after drying in the air, and the fuchsin solution is filtered over the preparation through a coarse filter paper in a funnel. The fuchsin is left on the slide for 4 minutes, is then washed off very rapidly with 0 5 per cent citric acid solution The citric acid solution is poured on and off, and the slide is very rapidly washed with tap water. It is then stained for about 10 seconds with a 1 per cent aqueous solution of methylene blue. With a little practice and adjustment to individual laboratory materials, this method gives excellent contrast stain; the intra- and extracellular rickettsiae are stained red, the cellular elements, blue[13] The stained smears are examined under the microscope, and those yolk sacs with satisfactory numbers of rickettsiae and no bacteria are selected for storage or for preparation of seed virus

7 *Preparation of seed* The seed for passage is prepared by thorough grinding of the infected yolk sacs either with hand mortar and pestle and sterile alundum or in a Waring blendor. If the blendor is used, extreme precautions must be taken to prevent laboratory infections Sufficient diluent is added to make a 10 per cent tissue suspension If the seed virus is to be stored, the diluent should be sterile skimmed milk, pH 7 2 (check pH before using) The frozen seed must be stored at a temperature of no less than -30° C and preferably lower. If the seed is to be used immediately, 0 85 per cent sterile saline solution is satisfactory as a diluent When the seed is to be inoculated into other eggs, it is usually diluted further (1-10, 1-100 or higher)

H. CULTIVATION OF RICKETTSIAE IN FERTILE EGGS

Since the rickettsiae have been cultivated only in the presence of living cells, various forms of tissue culture for different organisms have been described. The simplest and most satisfactory method is cultivation in the yolk sacs of embryonated eggs.[12]

1. *Preinoculation incubation.* The fertile eggs are removed from a 50° to 65° F. storage box and placed in an incubator with a temperature of 98 to 99.5° F. and humidity of about 65 per cent. The eggs should be turned at least twice a day during this pre-inoculation incubation. After 5 to 7 days of incubation, the eggs with viable embryos are then ready for inoculation.

2. *Source of material.* The usual source of material is blood removed from a guinea pig during its acute febrile illness. The blood is removed sterilely by cardiac puncture, cultured for possible contaminants, and inoculated immediately into the yolk sac of the fertile eggs It may be desirable to use suspensions of some of the organs, such as the spleen, spleen and liver, or brain.

3. *Preparation of material.* If a suspension of organs is desirable, they should be removed sterilely and should be ground with sterile sand or alundum. A suspension of approximately 10 per cent is prepared with sterile saline solution, is allowed to settle, and the supernatant used for the inoculation.

4. *Inoculation.* The eggs are marked by candling so that the location of the air sac and the embryo is clearly shown, the shell is sterilized with a solution of antiseptic, such as iodine or alcohol, a small hole is drilled in the air sac end of the egg with a dental drill, and the yolk sac is inoculated with a 1¼ inch to 1½ inch, 21 gauge needle, the needle directed away from the embryo Usually, .05 to 1 ml. of infectious material is placed in the yolk sac. The hole in the shell is then sealed with hot sterile paraffin.

5. *Incubation and candling.* After the inoculation, the eggs are incubated at 35° C.; the eggs are candled each day to determine the viability of the embryo. Infected yolk sacs harvested just before death of the embryos provide the greatest yields of rickettsiae.

6. *Harvest* When candling shows that the movements and blood circulation of the embryos are becoming sluggish, the yolk sacs should be harvested. There are many methods for harvesting, however, in small quantities, the following is suitable·

The shell is sterilized by washing in a suitable antiseptic, such as

TABLE 1
Isolation of Rickettsiae

Disease	Source	Preparation	Animals					Eggs	
			Animal	Routes	Quantity	Incubation	Signs	Quantity	Signs
Typhus fever epidemic	Human	Whole blood or macerated clot	Guinea pig	I P	3–5 ml	5–12	Fever	0 5–1 0 ml.	Sluggish movement, possible death Rickettsiae in smears of y. s.
			Cotton rat	I P or cardiac	1–3 ml 0 25–0 5 ml.		Illness in cotton rat		
	Lice	Ground							
Murine	Human	Whole blood or clot	Guinea pig	I P	3–5 ml.	3–10	Fever scrotal reaction	0 5–1 0 ml.	As above
	Flea	Ground or fed on rat	White rat		1–3 ml.				
	Rat brain	Macerated	White mouse		0 5–1 ml.		Inapparent in mouse		
Rocky Moutain spotted fever	Human	Whole blood clot	Guinea pig	I P	3–5 ml	3–10	Fever, illness scrotal reaction	0 5–1 0 ml.	Poor chance of primary isolation
	Ticks	Engorged and ground							
Q fever	Human	Whole blood clot or urine	Guinea pig	I P.	3–5 ml.	3–10	Fever	0 5–1 0 ml.	Sluggish movement; possible death Rickettsiae in smears of y s
	Ticks	Engorged and ground	White mice	I P	0 5–1 0 ml		Inapparent		
Rickettsialpox	Human	Whole blood (inoculated at bedside)	White mice	I. P	0 5–1 0 ml.	6–9	Ruffled fur inactivity	0 5–1 0 ml	Primary isolation has so far been unsuccessful in eggs
	Mites	Engorged and ground	Guinea pigs	I P	1–3 ml.	4–5	Fever and scrotal reaction		
	Mice	Brain or liver and spleen	Guinea pigs if mice are not infected with choriomeningitis	I P	3–5 ml.	4–5	Fever and scrotal reaction		
Tsutsugamushi disease	Human	Whole blood or clot	White mice	I. P	0 5–1 ml.	5–12	Fever, illness	0 5–1 0 ml	Poor chance of primary isolation
	Mites Rodent brains	Ground Macerated	Guinea pig		3–5 ml				

just before inoculation. The infectivity of whole yolk sacs are preserved well if the tissue is dropped into a small sterile vial, frozen in a mixture of solid carbon dioxide and alcohol, and stored at less than −30° C.

I. Immunologic and Serologic Identification of Strains

After one of the rickettsial agents has been isolated either in animals or eggs, a variety of procedures can be used for its identification. The serum of convalescent animals may be used for serologic studies by complement fixation, neutralization, and agglutination tests (see Part II). A few days after the animals have been bled for serum, they may be used for cross-immunity studies. The convalescent animals, together with suitable groups of negative and positive controls, are inoculated with known rickettsial strains and their immunity determined. These cross-immunity studies indicate broad immunologic relationships *only*; the finer degrees of variation are best studied by serologic technics The staining reactions, size, appearance, and growth characteristics are of some help in identifying an unknown rickettsia, but its serologic relationships are most important (see Part II). The successful applications of these methods will provide a positive identification of a known rickettsia or serve to establish the existence of new species.

J. Summary of Procedures for Isolation of Rickettsiae

Table 1 summarizes the procedures for the isolation of the rickettsiae in animals and in hens' fertile eggs. They can be isolated, as indicated in the table, from the blood of human cases and from the vector of the disease. In three instances, isolations are also possible from the rodent reservoir (for example, the rat in murine typhus, a variety of small rodents in tsutsugamushi disease, and the mouse in rickettsialpox). Laboratory animals other than those indicated may be used, but the ones listed are commonly employed for isolation studies at the National Institute of Health. Primary isolation of rickettsiae in embryonated eggs has not been very successful, although it has been accomplished in several instances. The usual procedure is to establish the strain in a susceptible animal and then transfer it to the yolk sacs of fertile eggs.

III. REFERENCES

See page 331.

TABLE 1
Isolation of Rickettsiae

Disease	*Source*	*Preparation*	Animals					Eggs	
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	Lice	Ground							
Murine	Human	Whole blood or clot	Guinea pig	I P	3–3 ml.	3–10	Fever scrotal reaction	0 5–1 0 ml.	As above
	Flea	Ground or fed on rat	White rat		1–3 ml				
	Rat brain	Macerated	White mouse		0 5–1 ml.		Inapparent in mouse		
Rocky Moutain spotted fever	Human	Whole blood clot	Guinea pig	I P	3–5 ml.	3–10	Fever, illness scrotal reaction	0 5–1 0 ml.	Poor chance of primary isolation
	Ticks	Engorged and ground							
Q fever	Human	Whole blood clot or urine	Guinea pig	I P.	3–3 ml.	3–10	Fever	0 5–1 0 ml.	Sluggish movement, possible death Rickettsiae in smears of y s
	Ticks	Engorged and ground	White mice	I P	0 5–1 0 ml		Inapparent		
Rickettsialpox	Human	Whole blood (inoculated at bed side)	White mice	I P	0 5–1 0 ml.	6–9	Ruffled fur inactivity	0 5–1 0 ml	Primary isolation has so far been unsuccessful in eggs
	Mites	Engorged and ground	Guinea pigs	I P	1–3 ml	4–5	Fever and scrotal reaction		
	Mice	Brain or liver and spleen	Guinea pigs if mice are not infected with choriomeningitis	I P	3–5 ml	4–5	Fever and scrotal reaction		
Tsutsugamushi disease	Human	Whole blood or clot	White mice	I. P	0 5–1 ml.	5–12	Fever illness	0 5–1 0 ml.	Poor chance of primary isolation
	Mites Rodent brains	Ground Macerated	Guinea pig		3–5 ml				

ISOLATION OF RICKETTSIAE

FRESH WHOLE BLOOD FROM EARLY FEBRILE PATIENT

INOCULATED INTO



NORMAL MALE GUINEA PIG

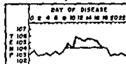


EPIDEMIC TYPHUS	MURINE TYPHUS	ROCKY MOUNTAIN SPOTTED FEVER



NO SCROTAL SWELLING





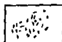


BRAIN



INOCULATED INTO YOLK SAC OF DEVELOPING CHICK EMBRYO



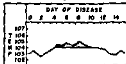


RICKETTSIAE IN YOLK SAC SMEARS



SCROTAL SWELLING







TUNICA





MOOSER BODIES CYTOPLASMIC RICKETTSIAE



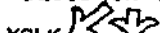
SCROTAL SWELLING AND NECROSIS







BLOOD OR SPLEEN



YOLK SAC



AGAR TISSUE CULTURE





INTRANUCLEAR RICKETTSIAE

AMDR 85-47

RICKETTSIAL DISEASES

PART II

Serologic Procedures for the Diagnosis of Rickettsial Diseases

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I. INTRODUCTION

INFORMATION concerning the immunologic reactions in rickettsial diseases has poured forth during the past few years. It resulted for the most part from the *confluence of two streams of development:* (1) the accumulated knowledge in the fields of bacteriology and immunology, and (2) the development of suitable methods for obtaining appreciable quantities of rickettsial organisms. The military importance of rickettsial diseases served as a third important tributary which greatly augmented the flow of accumulated facts during the war years. The recent advances have made methods readily available for the specific diagnosis of rickettsial diseases. The more important diagnostic technics will be discussed in detail in this chapter. In addition, other serologic reactions which are of value to the laboratory worker will be mentioned.

II. COLLECTION OF SPECIMENS

A. TIME

The serologic diagnosis of rickettsial diseases is established most convincingly by the demonstration of the appearance of specific antibodies in the blood of the patient, and their increase in titer during the course of infection and convalescence. Several types of antibodies of diagnostic importance develop at different times during the disease. Therefore, the well-equipped laboratory should perform several types of tests designed to provide the earliest possible tentative laboratory diagnosis and to provide the final specific laboratory diagnosis. Such a laboratory can be of most assistance to the clinician if he submits three samples of blood taken from the patient as follows. (1) during the *first few days of the illness, (2) during the 2d week, and (3)* toward the end of the 3d week after onset.

If the laboratory worker is to employ his procedures intelligently, he must know at what period the blood was drawn. Therefore, the clinician must indicate this temporal relationship on the request slip which accompanies each sample submitted. Some information of diagnostic value can be obtained from a single specimen of blood taken late in the febrile period or during convalescence, but such limited studies are not to be encouraged. It is the duty of the laboratory worker to educate the clinician to employ fully the available diagnostic tests to the best advantage for the patient, and this cannot be done when only one sample is submitted.

B. TECHNIC AND SHIPPING

Specimens for serologic studies are obtained and shipped as follows: Five ml of blood (no less) are drawn aseptically from the patient's vein with a sterile dry syringe and needle, placed in a sterile Wassermann tube, without anticoagulant, stoppered with a sterile cork or rubber stopper, and mailed to the laboratory in a double mailing container.* A request slip, bearing the patient's name, the physician's name and address, the clinical diagnosis, the date of onset of disease, the date on which the sample was taken, and the type of test requested, is wrapped around the tube of blood before it is placed inside the inner tin container. The outside cardboard container should bear the return address of the physician and the address of the laboratory; it should also be clearly marked "Rush—Specimen for Bacteriological Examination—Pouch with First-Class Mail" The attending physician should be cautioned against sending specimens so that they reach the laboratory on weekends or holidays It is preferable that he hold the specimen for a day or so in his refrigerator and then mail it to arrive on a work day

III WEIL-FELIX TEST

A. PREPARATION OF PROTEUS SUSPENSIONS AND TECHNIC OF THE TEST

The Weil-Felix test continues to be of considerable importance in the early presumptive diagnosis of a number of rickettsial diseases.[14] While it is recognized that the reaction is nonspecific, nevertheless, its simplicity warrants its use, especially in laboratories which are not equipped to make and use the specific rickettsial antigens The test tube titration of antibody is more accurate than the slide agglutination method, and the use of the latter is perhaps best restricted to field workers For details of the proteus slide agglutination technics of Castaneda [15] and of Welch,[16] the original articles should be consulted The procedures employed at the Army Medical School for the preparation of suspensions of *Proteus bacillus*, OX-19, OX-2, and OX-K strain, and their use in agglutination tests have been described by Plotz [17] as follows:

Only the O or non-motile variant of the *Proteus bacillus* is used for the agglutination reaction since it is this antigen which reacts specifically with sera from rickettsial diseases The purity of the strain should be controlled frequently by streaking on a veal agar infusion plate After twenty-four hours incubation, the smooth, non-spreading O type colonies are selected and transferred to tubes

* See Appendix concerning postal regulations

I. INTRODUCTION

INFORMATION concerning the immunologic reactions in rickettsial diseases has poured forth during the past few years. It resulted for the most part from the confluence of two streams of development: (1) the accumulated knowledge in the fields of bacteriology and immunology, and (2) the development of suitable methods for obtaining appreciable quantities of rickettsial organisms. The military importance of rickettsial diseases served as a third important tributary which greatly augmented the flow of accumulated facts during the war years. The recent advances have made methods readily available for the specific diagnosis of rickettsial diseases. The more important diagnostic technics will be discussed in detail in this chapter. In addition, other serologic reactions which are of value to the laboratory worker will be mentioned.

II. COLLECTION OF SPECIMENS

A. Time

The serologic diagnosis of rickettsial diseases is established most convincingly by the demonstration of the appearance of specific antibodies in the blood of the patient, and their increase in titer during the course of infection and convalescence Several types of antibodies of diagnostic importance develop at different times during the disease Therefore, the well-equipped laboratory should perform several types of tests designed to provide the earliest possible tentative laboratory diagnosis and to provide the final specific laboratory diagnosis Such a laboratory can be of most assistance to the clinician if he submits three samples of blood taken from the patient as follows (1) during the first few days of the illness, (2) during the 2d week, and (3) toward the end of the 3d week after onset.

If the laboratory worker is to employ his procedures intelligently, he must know at what period the blood was drawn Therefore, the clinician must indicate this temporal relationship on the request slip which accompanies each sample submitted. Some information of diagnostic value can be obtained from a single specimen of blood taken late in the febrile period or during convalescence, but such limited studies are not to be encouraged. It is the duty of the laboratory worker to educate the clinician to employ fully the available diagnostic tests to the best advantage for the patient, and this cannot be done when only one sample is submitted.

ings of agglutination of the OX-19 organism by sera from cases of epidemic and murine typhus, and of OX-K suspensions by sera from patients with scrub typhus are indicated. Similarly, the failure to obtain agglutination of any of the three organisms with sera of cases with Q fever is shown. The Weil-Felix response in Rocky Mountain spotted fever may be of several types, a high OX-19 and a low OX-2, or elevation of both, OX-19 and OX-2, or, occasionally, a low OX-19 and a high OX-2.

The Weil-Felix agglutinins may appear as early as the 5th or 6th day after onset of fever in those diseases in which the reaction becomes positive, and they are almost always present by the 12th day. These antibodies generally reach their maximum in early convalescence and then decline rather rapidly to nondiagnostic levels in one to several months Occasional patients with typhus or spotted fever do not develop OX agglutinins; this occurs more frequently in vaccinated persons who subsequently contract typhus.[19]

A rise in antibody titer, demonstrated in a series of two or more sera, is essential for the presumptive diagnosis of rickettsial disease when the Weil-Felix reaction is employed. One is not justified in interpreting the results of tests on a single serum, unless the titer is high, that is, well above 1/160. The Weil-Felix reaction is of no value in differentiating epidemic and murine typhus and frequently fails to provide even presumptive evidence for separating spotted fever from murine typhus.

The importance of maintaining a supply of known positive human sera for rechecking the bacterial suspensions at frequent intervals should be emphasized Aging sometimes renders such antigens hyperagglutinable; this should be suspected if a number of unexplained positive results are found.

IV SPECIFIC SEROLOGIC TESTS FOR DIAGNOSIS OF RICKETTSIAL INFECTIONS

The specific serologic technics of importance in the diagnosis of rickettsial infection are the complement fixation and agglutination tests which employ rickettsial material as antigen Although these methods were introduced a number of years ago,[20, 21, 22] it is only in the last few years that they have been employed frequently for clinical diagnostic work. Their extensive use had to await the development of methods for the preparation of materials rich in rickettsiae. Such

of dry agar and broth If the organism is non-motile the culture may be used for the agglutination test. All cultures should be maintained on dry agar slants Lyophilization is helpful in maintaining a culture as a pure O variant.

The agglutination reaction may be performed with living or killed cultures *The antigen is prepared by suspending eighteen to twenty-four hour agar cultures* in 0 85 per cent saline The turbidity of the suspension is adjusted to that of tube 3 of the McFarland nephelometer scale The killed antigen is prepared as follows. A smooth non-motile strain is grown on agar in Kolle flasks, and the culture is washed down with sufficient saline to make a heavy suspension, and 0 5 per cent formalin is added This represents the concentrated stock. This antigen is then diluted with saline when needed to have a turbidity equal to tube 3 of the McFarland scale.

A macroscopic agglutination test is performed by thoroughly mixing 0 5 cc. of serum dilution and 0 5 cc of antigen suspension Serum dilutions of 1 to 10 through *1 to 640 (final dilution 1 to 20 through 1 to 1280)* are *usually sufficient* A control tube containing 0 5 cc of antigen and 0 5 cc. of saline should be included as well as a positive serum control Tests and controls are incubated in the water bath at 37° C for two hours followed by storage overnight in the ice box Complete agglutination is indicated by absolute clearing of the supernatant fluid and by settling of the organisms in large white particles at the bottom of the tube Partial agglutination is indicated by incomplete clearing of the supernatant fluid and diminution in size of the bacterial clumps When the tubes are shaken, granular agglutinated masses of bacteria are seen

TABLE 1

Usual Weil-Felix Agglutination Reactions Observed in Rickettsial Diseases

	OX-19	OX-2	OX-K
Epidemic Typhus	++++	+	0
Murine Typhus	++++	+	0
Scrub Typhus	0	0	++++
Q Fever	0	0	0
Rocky Mountain Spotted Fever	++++	+	0
	+	++++	0

From H Plotz [18]

B. INTERPRETATION OF RESULTS

The typical responses obtained with suspensions of OX-19, OX-2, and OX-K Proteus organisms and convalescent sera from cases of epidemic, murine, and scrub typhus, Rocky Mountain spotted fever, and Q fever are given in Table 1. This table was modified by Plotz [18] from a similar chart prepared by Felix [14] some years ago. The classical find-

areas in which it has been demonstrated that only one type of typhus occurs, it is feasible to use the simpler, less expensive antigen of Bengtson for the diagnosis of typhus.

In the absence of other types of complement-fixing antigen, commercial typhus vaccine has been used for testing sera[30,31] This procedure has great limitations since the vaccine antigen will not differentiate between epidemic and murine typhus, and will react with Wassermann positive sera, however, it can be employed in an emergency until better antigens can be furnished.

2. Preparation of epidemic and murine typhus antigens. Only those methods employed in the preparation of epidemic and murine typhus antigens from infected yolk sac tissue will be described in detail at this time. The preparation of rickettsial suspensions from lungs of rodents infected by the intranasal route will not be discussed because of the great hazard of infection to laboratory personnel which results when this technic is used. Although this danger can be minimized if elaborate precautions[32] are taken, these are beyond the reach of the ordinary laboratory.

During the past few years the technics employed by Bengston and her coworkers have gradually undergone change[33] and only that currently employed at the National Institute of Health will be described Method A, used by Craigie[34] in making his earlier typhus vaccine and providing the starting material for the preparation of antigen of the Plotz type is as follows·

The method yields stable suspensions of epidemic or murine rickettsiae that effectively protect guinea pigs against considerable multiples of the minimum infective dose of infective rickettsiae.

At the time of collection, the infected yolk sacs are allowed to drain on bronze screens but no attempt is made to wash adhering yolk from them The yolk sacs are triturated by shaking in heavy glass bottles with glass beads, and the following diluent is used in the ratio of 5 or 10 cc per yolk sac

0 85% sodium chloride in distilled water	4 parts
Sorensen's sodium potassium phosphate buffer, pH 7 0	1 part
Formalin (40% formaldehyde)	to 0.5%

The suspension is filtered through bronze gauze and placed in the cold room to permit the yolk cream to rise to the surface The suspension is siphoned off from below the yolk layer and centrifuged at 4000 r p m in the refrigerator to deposit the rickettsiae along with other particulate matter present The floating yolk cake is carefully removed and the supernatant is discarded Care is taken to swab adhering traces of yolk from the tube before proceeding to resuspend the deposit The deposit is resuspended in diluting fluid similar in composition to that noted above The diluting fluid is added in the proportion of 10 cc per

methods became available when Castaneda [23] showed that rodent lungs were suitable for the profuse growth of *R. mooseri*, when Cox [12] demonstrated the usefulness of the yolk sac tissue of embryonated eggs for the culture of the typhus and spotted fever groups, and when Zinsser, Fitzpatrick, and Wei grew *R. prowazeki* on their tissue agar medium.[24] In general, the complement-fixation test has been employed more widely than the agglutination test, but each technic is satisfactory in the hands of those experienced with it.

A. COMPLEMENT FIXATION

1. *General remarks.* Specific complement-fixing antibodies are demonstrable in convalescent sera from patients with epidemic, murine and scrub typhus, spotted fever (Rocky Mountain, Mexican), boutonneuse fever, South African tick bite fever, North Queensland tick typhus, Q fever, and rickettsialpox Certain of these rickettsial agents have antigens which are closely related, for example, the soluble antigen of epidemic and murine typhus [2, 25, 26, 27] and the soluble antigen of spotted fever and boutonneuse fever.[3] Therefore, a complement-fixing antigen which contains the rickettsiae of epidemic typhus together with large amounts of the soluble antigen common to epidemic and murine organisms can be used to diagnose infection with either of these agents, but such antigen is not suitable for differentiating between epidemic and murine typhus Highly specific antigens which will make such a differentiation possible can be obtained by eliminating all of the soluble substance from the rickettsiae of epidemic typhus; this is done by repeatedly washing the rickettsial organisms Similarly, suspensions of washed rickettsiae of murine typhus can be prepared and used for specific diagnosis. It may be pointed out that the original complement-fixing antigens of epidemic and murine typhus which were prepared by Bengtson and Topping [28] frequently contained appreciable amounts of the soluble substance On the other hand, the thoroughly washed rickettsial organisms which Plotz and his coworkers [29] used were essentially free of the soluble substance These latter highly purified rickettsial antigens are relatively expensive, whereas the less thoroughly washed antigens are comparatively cheap. If it is important to know whether epidemic or murine infection has occurred in a given patient, or in a given area, then the purified suspensions should be used, in military medicine these points were of vital importance, and the antigens of Plotz were employed in the Army. On the other hand, in

3. *Complement fixation technic.* The complement-fixing technic used at the Army Medical School is as follows:[29]

The complement titration is carried out with varying quantities, i e, 0 08 ml to 0.26 ml. with increments of 0 02 ml of a 1/30 dilution of commercial lyophilized guinea pig serum The hemolytic system consists of equal parts of 3% washed sheep erythrocytes and 3 minimal hemolytic doses (MHD) of anti-sheep erythrocyte hemolysin The titration is performed as follows

Tube No.	1	2	3	4	5	6	7	8	9
Complement 1/30	0 08 ml	0 10	0 12	0 14	0 16	0 18	0 20	0 22	0 24
Saline 0 85%	0 67 ml	0 65	0 63	0 61	0 59	0 57	0 55	0 53	0 51
Antigen 2 units	0 25 ml	0 25	0 25	0 25	0 25	0 25	0 25	0 25	0 25
Sensitized R B C	0 50 ml	0 50	0 50	0 50	0 50	0 50	0 50	0 50	0 50

Incubate 37° C for 30 minutes

The first tube showing complete hemolysis is taken as the exact unit and the next higher amount as a full unit Two full units of complement determined in the presence of antigen and contained in 0 5 ml of 0 85% saline are used in the test

The serum to be tested is inactivated at 56° C. for 30 minutes 0.25 ml amounts of antigen, at a dilution which represents 2 units,* are mixed with 0 25 ml volumes of serial two-fold dilutions of serum, and then 0 5 ml of diluted complement is added The mixtures are incubated at 4° C. overnight. The following morning 0 5 ml of the sensitized cells are added The test materials are incubated at 37° C for 30 minutes, when final readings are made Titration end-points are estimated on the basis of the last tube showing complete or 3 plus fixation, and the titer is taken as the dilution of serum originally added to that tube In each test, known positive and negative sera are titrated to end-points with two units of a previously standardized antigen This titration serves as a control on the activity of the hemolytic system and permits a comparison of the data obtained from one experiment to another. In addition, a complement titration in the presence of antigen is included in each test to determine the amount of complement available after incubation.

The titration of complement is carried out in a similar manner at the National Institute of Health[8, 86] except that 0 2 ml. volumes of diluted complement are used and 0 4 ml. amount of a suspension of sensitized sheep cells (which consists of equal parts of a 2 per cent suspension of R B C. and solution of hemolysin so diluted that each 0 2 ml. contains two units, the two being mixed thoroughly 10 minutes prior to use). Sera to be tested are inactivated at 56° C. for ½ hour and 0 2 ml amounts of serial 2-fold dilutions prepared in saline solution The test is then performed as follows[85]

To the serum dilutions contained in the tubes are added 0 2 cc of the proper dilution of antigen (4 units) † and 0 2 cc of complement (2 units).

* An antigen unit is the smallest amount which gives a complete *fixation* with 2 full units of complement in the presence of 4 units of antibody

† Two units of antigen are now being used instead of 4.

yolk sac represented by the deposit. The deposit, after thorough dispersion, is kept in the refrigerator for several days to permit sedimentation of coarse particles to occur. The supernatant, which contains rickettsiae in suspension, is processed thus

A separatory funnel of suitable capacity is half-filled with the suspension and a half-volume of ethyl ether is added The funnel is shaken vigorously and extensive emulsion formation occurs. If separation of ether at the upper surface does not begin within a few minutes, a further amount of ethyl ether should be added and incorporated by light shaking In a short period on standing, provided care has been taken to exclude the light fraction of yolk, separation occurs into three layers (a) an upper layer of excess ether, (b) a middle emulsion layer, and (c) a lower aqueous layer. The middle emulsion layer (b) contains tissue fragments and yolk particles while the lower aqueous layer (c) contains rickettsiae in suspension along with a reduced amount of cell debris

At the Army Medical School the partially purified suspensions of *R. prowazeki* and of *R mooseri* obtained by Craigie's technic were subjected to additional treatment by Plotz [29] as follows:

The lower aqueous layer is centrifuged in the cold in an angle centrifuge at 4000 r p m for one hour The supernatant fluid contains a soluble antigen which is capable of fixing complement in the presence of both epidemic and murine convalescent serum The sediment is resuspended in 1/10th the original volume of saline . . and . . again extracted with ½ volume of ethyl ether The aqueous suspension is drawn off and again centrifuged in the cold in an angle centrifuge at 4000 r p m. for one hour The supernatant is discarded and the sediment is washed similarly four times in buffered saline After each centrifugation, the sediment is easily suspended by shaking with glass beads The final sediment is suspended in 0 2% formol saline to a volume of 50 ml per 300 yolk sacs

Epidemic (Breinl strain) and murine (Wilmington strain) antigens of the Plotz type are now prepared for the Army Medical School by Parke, Davis and Company; they titer in the neighborhood of 1/200 and generally are used in diagnostic tests at a 1/100 dilution.

The most recent method employed by Topping and Shepard [35] at the National Institute of Health treats infected yolk sac material as follows·

the homogenous mass which results from thorough grinding in a blendor can either be made into a 10-per cent emulsion with saline (which may or may not contain formalin) . is shaken directly with 1½ volumes of diethyl ether [4] The emulsion soon breaks into three distinct phases—a clear yellow ether at the top, an interphase of extraneous material containing some rickettsiae, and the aqueous phase which, with some of the strains, contains most of the rickettsiae plus the soluble antigen released from the rickettsiae by exposure to the ether If it is desired to separate the rickettsiae from the soluble antigen, this can be done by centrifugation of the aqueous phase

flasks are stoppered with rubber plugs, and incubated at 35° C. for 7 days; any flasks showing bacterial colonies are discarded at this time Fluid of condensation is pipetted from each flask and discarded Five ml. of physiological saline solution, pH 7 5, containing 0 2% U.S.P formaldehyde are added to each flask and the surface growth is gently scraped into the fluid with a glass pusher; the cell suspension is then removed from the flask by a large bore pipette

Material from 20 to 40 Kolle flasks is pooled in a pyrex centrifuge bottle containing glass beads and shaken on a machine for 15 minutes The bottle is centrifuged at 2000 r p m. for 10 minutes and the supernatant fluid removed and saved The bottle containing the moist sediment and beads is restoppered and reshaken on the machine, then the material is taken up in 40–50 ml of diluent and centrifuged at 2000 r p.m for 10 minutes The supernatant fluid is added to the first lot and the sediment is discarded Then the pooled supernatants are stored at 5° C. for 3 to 8 days to allow time for the material to become non-infectious The rickettsiae are sedimented from the pooled fluids by centrifugation at 4500 r.p.m in 50 ml pyrex tubes in a refrigerated angle centrifuge for one hour. After the supernatant fluid has been removed and discarded, the sediment is carefully resuspended by constant rubbing with a rubber tipped rod while formol-saline solution to half the original volume is added slowly, drop by drop at first. The resuspended sediment is shaken with a half volume of ethyl ether in a separatory funnel, and the aqueous phase is removed after half an hour at room temperature. The rickettsial material is washed once or twice more by differential centrifugation, and finally resuspended in formol-saline solution to 1/10th the original volume, and merthiolate is added to a concentration of 1/10,000 Gross particles are removed by low speed centrifugation and the antigen is ready for use. Such preparations have titration end-points of 1/16 to 1/32 and are used in diagnostic tests at dilutions of 1/8 to 1/16

It should be pointed out that thoroughly washed rickettsial antigens of epidemic and murine typhus contain only minute amounts of egg protein [41] and are free of the Wassermann antigen, moreover, they are generally used in high dilutions On the other hand, Rocky Mountain spotted fever antigens prepared from infected yolk sacs usually are less thoroughly freed of extraneous material and are used in low dilution. Recent studies [42] indicate that from 20 to 80 per cent of soldiers who have been repeatedly immunized with vaccines prepared from embryonated eggs (typhus, yellow fever, influenza, and Japanese encephalitis) have antibodies which fix complement with antigens prepared from suspensions of normal chick tissue by high speed centrifugation (14,000 r p m.); the per cent of positive reactions varies with length of time following the last vaccination Difficulties which probably arise from this fact have led to the continued use at the Army Medical School of tissue culture antigens for the diagnosis of spotted fever. Even though such preparations are relatively clean, it is the

After 1 hour's incubation in the 37° water bath the sensitized sheep cells are added in 0 4 cc. amounts, the hemolysin (2 units per 0 2 cc) and 2 per cent sheep cells having been prepared by thorough mixing 10 minutes previously. After further incubation in the 37° water bath for 1 hour, the test is placed at the cold room temperature and read the following morning Positive results are recorded as 4, 3, 2, 1+, and trace, and the titer is read as the highest dilution showing 3 or 4+ fixation.

The following controls are set up:

Serum controls. To duplicate tubes of the four lowest dilutions are added the same reagents as for the test except that salt solution is substituted for antigen.

Antigen controls. Antigen controls contain twice the volume of the dilution used in the test, i e, 0 4 cc, and 0 2 cc of complement and 0.4 cc of sensitized cells

Hemolytic system The hemolytic system control consists of four tubes containing 0 05, 0 1, 0 15, and 0 2 cc. of the dilution of complement used in the test, these amounts representing ½, 1, 1½, and 2 units The volume in each tube is made up to 0 6 cc. with sterile saline, and 0 4 cc. sensitized cells added The tube containing 0 05 should show 1 or 2+ fixation, and the three remaining tubes should be completed hemolyzed.

Standard serum. A standard serum composed of a pooled lot of serums from recovered guinea pigs is titrated with each test, using the same dilutions as for the serums under test

4. *Spotted fever group including rickettsialpox.* Included in the spotted fever group of rickettsiae are the Rocky Mountain, Brazilian, Colombian, and Mexican strains of *Dermacentroxenus rickettsi*, the agents of boutonneuse fever and of South African tick bite fever, and the newly described agent of rickettsialpox The inter-relationships of the organisms have been reviewed recently elsewhere.[37] Certain of the organisms in this group grow as profusely in yolk sac tissue as do *R. prowazeki* and *R mooseri*, and the methods used for the preparation of typhus antigens can be applied. Bengtson [38] made diagnostic antigens for Rocky Mountain spotted fever in this manner, and Huebner and Armstrong prepared antigens of rickettsialpox.[5] Moreover, Cox and his associates [39] now prepare such antigens by defatting lyophilized infected yolk sacs. Complement-fixing antigens for the diagnosis of spotted fever have been prepared at the Army Medical School for a number of years from tissue agar material The original method described by Plotz and Wertman [40] has been modified and now is as follows:

Kolle flasks containing the agar medium of Zinsser, Fitzpatrick, and Wei [34] are each inoculated with 2 ml. of a mixture consisting of two parts of minced chick embryo cells and one part of infectious material consisting of a 10% suspension of yolk sac tissue which is as rich in rickettsiae as can be obtained. The

derscheer, Clancy, and Cox,[39] 50 per cent suspensions of infected yolk sac are prepared in distilled water containing 0 8 per cent phenol and stored at 4° C. for 48 hours to allow time for the suspension to become noninfectious. The suspension is then lyophilized and the dried material pulverized. It is then extracted at 4° C. with several changes of ether over a period of hours. The ether is removed by high vacuum and the powder resuspended in sufficient saline solution containing 0.3 per cent phenol to equal the volume of the original tissue suspension. The suspension is shaken occasionally during storage at 4° C. for the next 3 to 5 days. It is then centrifuged in an angle machine at 3,000 r p m for 30 minutes and the resultant supernatant fluid serves as antigen

7. *North Queensland tick typhus.* Complement-fixing antigens which react with antibodies against the newly recognized agent of North Queensland tick typhus have been prepared from infected agar tissue cultures[41] and yolk sacs.[42]

B. AGGLUTINATION

1. *General remarks.* Rickettsial agglutination tests are quite satisfactory for diagnostic purposes in epidemic and murine typhus[29, 50, 51, 52, 53] and in Q fever.[54] Antigens for this work usually are prepared from infected yolk sac tissue, but suspensions of typhus organisms are also obtained from infected mouse lungs. Slide technics[15, 55, 56] can be used for rickettsial agglutination, but in the author's opinion, a test tube method is preferable. Technical difficulties which prevent the easy preparation of rich, purified suspensions of rickettsiae are undoubtedly responsible for the failure to employ this technic more extensively with members of the spotted fever group or in North Queensland tick typhus and scrub typhus; however, it has been used in Rocky Mountain spotted fever.[21, 57, 58]

2. *Macroscopic technic.* The macroscopic rickettsial agglutination technic of Plotz and his associates[29] has been used at the Army Medical School for the past several years It employs purified suspensions of *R. prowazeki* and *R mooseri* prepared in the manner described above for complement-fixing antigens The agglutinating suspensions used in the test represent a 1/6 to 1/15 dilution of a stock suspension of rickettsiae Thus, the antigen is used in a more concentrated form in the agglutination test than in the complement fixation technic, and each tube in the test receives the equivalent of about 10 complement-fixing units of antigen The authors describe[29] their procedure as follows:

custom to employ an additional control with each test serum. This is set up like the anticomplementary control with the lowest dilution of serum and contains 0.2 ml. of normal chick embryo tissue antigen preserved in the same way as the spotted fever antigen.

5. *Q fever.* The complement fixation test has proved extremely useful in the diagnosis of Q fever.[43] Some antigenic variation exists in the different strains of *R. burneti*, and certain of these provide much broader complement-fixing antigens than others.[44, 45] The Henzerling strain of Q fever [44] recovered in Italy is one of the best for the preparation of diagnostic antigens. Infected yolk sacs are used as starting material and are processed by methods similar to those described above for typhus antigens. A soluble substance has not been demonstrated in Q fever antigens.[35]

6. *Scrub typhus.* Complement-fixing antigens of scrub typhus have been prepared from infected yolk sacs [46] and from infected rodent tissue.[47] While the antigens associated with the various strains of scrub typhus are distinct from the other rickettsial diseases mentioned, it is to be noted that certain strains of *R. orientalis* differ considerably from others in their antigenic pattern [46] This limits the usefulness of the test since a number of antigens prepared from different strains must be used for testing each serum

Two recently described methods for preparing scrub typhus antigens from infected yolk sac tissue differ from the usual technic employed for making epidemic typhus antigen. The procedure of Topping and Shepard [35] is as follows:

> The ground yolk sacs are placed in 500 cc centrifuge bottles, and 10 volumes of cold ether is added The mixture is thoroughly shaken in the cold (4° C.) for 30 minutes to 1 hour The ether becomes very yellow and on standing a few moments a reddish mass of tissue falls to the bottom of the bottle. The ether is decanted as completely as possible and the tissue washed with cold ether until no yellow color is visible in the ether Usually once or twice is sufficient Approximately 1 cc. of sterile distilled water is added for each gram of yolk sac, and the mixture is thoroughly shaken The ether in solution is removed under a partial vacuum and the tissue suspension allowed to stand overnight in the refrigerator The next morning it is centrifuged at about 3000 r p m for 20 to 30 minutes . . The clear red supernatant is a satisfactory antigen

It should be noted that in this method the material is infectious when originally treated with ether. While ether inactivates the organism, extensive care must be employed during the early steps to avoid infection of laboratory personnel. In the method of Wolf, Van-

derscheer, Clancy, and Cox,[39] 50 per cent suspensions of infected yolk sac are prepared in distilled water containing 0 8 per cent phenol and stored at 4° C. for 48 hours to allow time for the suspension to become noninfectious. The suspension is then lyophilized and the dried material pulverized. It is then extracted at 4° C. with several changes of ether over a period of hours. The ether is removed by high vacuum and the powder resuspended in sufficient saline solution containing 0 3 per cent phenol to equal the volume of the original tissue suspension The suspension is shaken occasionally during storage at 4° C. for the next 3 to 5 days. It is then centrifuged in an angle machine at 3,000 r.p m for 30 minutes and the resultant supernatant fluid serves as antigen.

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formalin, and about 0.3 g of Celite added (Celite Analytical Filter Aid, Johns-Manville) for each initial gram of yolk-sac When the mixture is shaken flocculation of the Celite occurs, and the Celite may be removed by a few minutes centrifugation at about 1800 r p m. Because of the loose nature of the sediment it is preferable to pour off the supernate rather completely and repeat the slow centrifugation The resulting supernate is then centrifuged at 3600 r p m for an hour and the sediment is resuspended in the desired volume of a solution containing 0.85 per cent sodium chloride, 0 01 M phosphate buffer at pH 7 0, and 0 1 per cent formalin Smears of the final preparations stained by the Macchiavello technic show nearly pure preparations of the rickettsiae with an occasional small nucleus from yolk-sac tissue which stains blue.

The rickettsial suspensions are used in agglutination tests by the authors in the following manner.

0.2 ml of rickettsial suspension of the desired turbidity are mixed with 0.2 ml of suitable dilutions of antiserum, and the mixture placed in a 5 mm diameter tube. After incubation at 37 C for 4 hours the tests are placed in the cold room overnight and the results recorded next morning + + + + has been used to represent complete agglutination, + as agglutination visible only with a hand lens, and O as no observable reaction.

Two points should be mentioned During the early stage of processing antigen, the material is infectious and must be handled carefully However, the authors state (unpublished) that formalinized suspensions of yolk sac which have been stored sufficiently long to become non-infectious can be processed satisfactorily by the above method. Suspensions of epidemic and murine organisms prepared in this manner give considerable crossing when tested with sera from guinea pigs or rabbits convalescent from epidemic or murine typhus

3. *Slide technics* Castaneda [59] prepared, from lungs of intranasally infected mice, suspensions of washed *R prowazeki* and *R mooseri* which had a turbidity corresponding to 15 mm. of the Gates loop Lots of 20 mice usually yielded 5 to 10 ml of antigen Dilutions of human serum to be tested were prepared in test tubes covering the range from 1/80 to 1/640 Drops of antigen were placed on an ordinary slide with a bacterial drop of 4 mm diameter, and the dilutions of serum added with the same loop beginning with the highest and progressing to the lowest dilution The slides containing the mixtures were arranged in petri dishes, each containing a piece of moist cotton, and placed on a phonograph plate which rotated at 15 r p m and was held on a 15° inclination Readings were made in 5 to 10 minutes The results obtained in slide agglutination tests were comparable to those obtained in complement fixation tests in which the same antigens and sera were

After various attempts to devise a method to give more reproducible end-points, it was found that the use of conical tubes promoted a greater aggregation of particles and greatly facilitated reading. While smaller tubes may be used, it has been found that the 3 cc. conical pyrex centrifuge tubes (Corning Glass Works, Catalog No. 8060) measuring 10 mm × 65 mm. have given optimal results

Using these tubes, the aggregation of rickettsiae was firm and not easily dispersed It was not necessary to centrifuge the tubes or to examine stained preparations in order to demonstrate agglutination. The test was extremely easy to read, especially when examined by artificial light. When the tests were read by two or more observers, comparable endpoints were recorded

. . . One technical detail of importance must be noted It was necessary to add normal human serum in sufficient quantity to give a concentration of 1:200 (0 5%) in the antigen preparation to be used This small amount of serum was sufficient to prevent spontaneous agglutination of the rickettsiae

Serial dilutions of serum were made in physiological saline and distributed in 0 25 cc amounts To each dilution of serum was added 0 25 cc of rickettsial suspension. The mixture was then thoroughly shaken and placed in a water bath at 42° C. for 4 hours followed by storage in the ice box at 4° C for another 16 to 18 hours when the test was read A test was recorded as "complete" agglutination when the clumps had settled to the bottom of the tube, leaving a clear supernate, while a "partial" agglutination was one where definite clumping had occurred and settled out, but the supernate remained slightly cloudy. Only complete and partial agglutinations were recorded as positive and all titers represented final dilution Positive and negative controls were included with each test.

Shepard and Topping [58] have described still another method for processing infected yolk sacs which yield suspensions of *R. prowazeki, R. mooseri, D. rickettsi*, and *R. burneti* suitable for use in a macroscopic agglutination test. This depends upon the use of Celite for the absorption of extraneous material and was derived from a technic which Fulton and Begg [26] developed for purification of suspensions of *R. prowazeki* and *R. mooseri* from infected rodent lungs The details are as follows:

Yolk-sacs infected with a particular rickettsia are weighed and placed in a blendor with 10 ml of 0 1 M phosphate buffer at pH 7 65 with 0 1 per cent formalin, per gram of yolk-sac After grinding, the preparation is centrifuged at 3600 r p m. for one hour in an angle centrifuge, and the supernate is discarded When the supernate is poured off, the tubes are placed on their sides, and the solidified fat which usually clings to the walls of the tubes is wiped out with cotton The sediment is then resuspended to volume in distilled water containing 0 1 per cent formalin by repeated aspiration and forceful expulsion against the bottom of the tube with a pipet Two more similar centrifugal sedimentations and resuspensions of the rickettsiae usually result in a preparation which sediments completely leaving a clear supernate After the third resuspension the volume is made up to twice the original volume with water containing 0 1 per cent

formalin, and about 0.3 g of Celite added (Celite Analytical Filter Aid, Johns-Manville) for each initial gram of yolk-sac. When the mixture is shaken flocculation of the Celite occurs, and the Celite may be removed by a few minutes centrifugation at about 1800 r p m Because of the loose nature of the sediment it is preferable to pour off the supernate rather completely and repeat the slow centrifugation The resulting supernate is then centrifuged at 3600 r p.m for an hour and the sediment is resuspended in the desired volume of a solution containing 0.85 per cent sodium chloride, 0 01 M phosphate buffer at pH 7 0, and 0 1 per cent formalin. Smears of the final preparations stained by the Macchiavello technic show nearly pure preparations of the rickettsiae with an occasional small nucleus from yolk-sac tissue which stains blue.

The rickettsial suspensions are used in agglutination tests by the authors in the following manner:

0.2 ml of rickettsial suspension of the desired turbidity are mixed with 0.2 ml. of suitable dilutions of antiserum, and the mixture placed in a 5 mm diameter tube After incubation at 37 C for 4 hours the tests are placed in the cold room overnight and the results recorded next morning + + + + has been used to represent complete agglutination, + as agglutination visible only with a hand lens, and O as no observable reaction

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TABLE 2

Summary of Specific Serologic Tests in Rickettsial Diseases of Man

Group	Disease	*In Vitro* Tests			*In Vivo* Tests			Usual Test Animal
		Type	Common Sources of		Type	Common Sources of		
			Antigen	Antiserum		Virus	Antiserum	
Rickettsiae	Epidemic Typhus	*Complement fixation*	*Yolk sac* *Mouse lung*	*Man* Guinea pig	Neutralization	Guinea pig brain	Guinea pig Monkey Man	Guinea pig Cotton Rat
		Agglutination	*Yolk sac* *Mouse lung*	*Man* Rabbit Guinea pig	Antitoxin	Yolk sac	Guinea pig Man	Mouse
		Precipitin	Yolk sac	Man Rabbit Guinea pig				
	Murine typhus	*Complement fixation*	*Yolk sac* *Rat lung* *Mouse lung*	*Man* Rat Guinea pig	Neutralization	Guinea pig tunica Yolk sac	Guinea pig Man	Guinea pig *Mouse* Cotton rat
		Agglutination	*Yolk sac* *Rat lung* *Mouse lung*	*Man* Rabbit Guinea pig	Antitoxin	Yolk sac	Guinea pig Man	Mouse
		Precipitin	Yolk sac	Man Rabbit Guinea pig				
	Rocky Mountain spotted fever	*Complement fixation*	*Yolk sac* *Agar tissue culture*	*Man* Guinea pig	Neutralization	Guinea pig blood	Guinea pig Man	Guinea pig
		Agglutination	Agar tissue culture Yolk sac	Rabbit Man Guinea pig				
	Boutonneuse fever	*Complement fixation*	*Yolk sac*	*Man* Guinea pig				

TABLE 2—*Continued*

Summary of Specific Serologic Tests in Rickettsial Diseases of Man

Group	Disease	*In Vitro* Tests			*In Vivo* Tests			Usual Test Animal
		Type	Common Sources of		Type	Common Sources of		
			Antigen	Antiserum		Virus	Antiserum	
Rickettsiae	South African tick bite fever	*Complement fixation*	*Yolk sac*	Guinea pig				
	Scrub typhus	*Complement fixation*	*Yolk sac* Mouse lung Rat lung	*Man* Guinea pig Mouse	Neutralization Antitoxin	Yolk sac Mouse spleen Yolk sac	Man Guinea pig Rabbit Guinea pig Rabbit Man	Mouse Mouse
	Q fever	*Complement fixation* *Agglutination*	*Yolk sac* Mouse lung *Yolk sac* Mouse spleen	Man Guinea pig *Man* Guinea pig Rabbit Bandicoot Mouse	Neutralization	Guinea pig spleen or blood Yolk sac	Guinea pig Man	Guinea pig
	North Queensland tick typhus	*Complement fixation*	*Agar tissue cultures* *Yolk sac*	*Man* Guinea pig				
	Rickettsialpox	*Complement fixation*	*Yolk sac*	*Man* Guinea pig				

Technics and sources of antigen which are italicized in table are commonly used for diagnosis of human diseases.

used. Differentiation between epidemic and murine typhus was apparently demonstrated by both technics but at the time Castaneda seemed hesitant about relying on either technic to differentiate between epidemic and murine typhus in man.

Fitzpatrick[65] prepared suspensions of *R. prowazeki* and *R. mooseri* from infected yolk sacs by a technic similar to Method A of Craigie (see Section IV, A, 2, above). Dilutions of the serum to be examined were made in test tubes, and one drop of each dilution was transferred with a capillary pipette to the depressions in a glass slide. An equal amount of rickettsial suspension was added to each concavity, and the slides were rotated by hand and placed on moist paper in a petri dish. The dishes were held in an incubator at 40° C. for 5 hours and then were kept overnight in a refrigerator Readings were made with the aid of a hand lens Cross-agglutination occurred with convalescent human sera and the two antigens, but somewhat higher titers were obtained with the homologous antigen.

C. INTERPRETATION OF RESULTS OF COMPLEMENT FIXATION AND AGGLUTINATION TESTS

In the diagnosis of rickettsial diseases, as elsewhere it is essential to demonstrate a rise in specific antibodies during convalescence If this is done, it is unnecessary to quibble about the significance of positive results obtained with low dilutions of serum However, since serial specimens are not always received, it is necessary to set certain minimal levels as having significance in a single test. In general, a titer of 1/10 (original dilution of serum) is significant in complement fixation tests in which washed rickettsial antigens are used. At some time during convalescence in most of the rickettsial diseases specific complement fixation titers well above 1/200 are usually obtained Rickettsial agglutination titers above 1/25–1/40 are probably significant in epidemic and murine typhus, while titers greater than 1/5 have been considered of diagnostic value in Q fever.

The interpretation of the results of rickettsial complement fixation and agglutination tests rests on more extensive knowledge in epidemic and murine typhus than in the other rickettsial diseases The sera of individuals who have been repeatedly injected with epidemic typhus fever vaccine generally show complement fixation titers of 1/4–1/32 with epidemic antigen Such individuals do not develop increased titers when they suffer from febrile illnesses of nontyphus etiology.[66] How-

ever, when such vaccinated persons become infected with either the homologous or heterologous typhus organism, they show a prompt rise in epidemic and murine complement-fixing antibodies.[19, 37, 61] The amount of crossing in such tests may be sufficient to preclude a differential diagnosis between epidemic and murine typhus Nevertheless, a serologic diagnosis can generally be made in these individuals by means of the rickettsial agglutination test, since higher titers are obtained with antigens prepared from the infecting organism

V. SPECIFIC RICKETTSIAL SEROLOGIC TESTS OF LABORATORY IMPORTANCE

There are several types of serologic tests which are of assistance to the laboratory worker in carrying out various fundamental studies, but which are of little importance in the diagnosis of human disease. Among these are the antitoxin titration, the neutralization test, and the precipitin reaction. Needless to say, the complement fixation and agglutination technics are as valuable in investigative work as they are in clinical diagnosis

A Antitoxin Titration

A toxic substance, capable of causing death of mice within a few hours, is found in yolk sacs which are rich in the organisms of murine,[62] epidemic,[63, 64] and scrub typhus[65] The toxins of epidemic and murine typhus are closely related but not identical,[66, 67] whereas the toxin of scrub typhus bears no relation to the two just mentioned[65] The sera of men and animals recovered from infection with these agents contain immune substances which are capable of neutralizing the toxic effect[62, 63, 64, 65, 66] While the demonstration of antitoxin in the serum of convalescents might be used for the diagnosis of typhus, this type of test has not had wide application because of the technical difficulties associated with it Nevertheless, the antitoxin test played a considerable role in the development of typhus vaccines[25, 68] and is still used in the standard assay of epidemic typhus vaccine[69]

B. Neutralization Tests

Neutralization tests or protection tests have been used with most of the rickettsial agents, the principle here is the same as in neutralization tests employed with viral agents Thus, antibody is added to a suspension of organisms, and the mixture is injected into susceptible

animals. If the animals fail to show evidence of infection, the organism is said to be neutralized by the antibody. In general, the results obtained in this technic are not as clean-cut as with some of the viral agents, and while the test is useful under certain conditions, it is not employed too frequently because other simpler methods are available which usually may be substituted for it.

C. PRECIPITIN REACTION

The precipitation reaction has been used in certain of the studies with soluble antigens of rickettsiae[70] where complement fixation was not readily applicable One of the limitations of the precipitation method in rickettsial work is the difficulty in obtaining relatively pure soluble antigens, it will be recalled that rickettsiae only grow in the presence of living tissue, and the occurrence of large amounts of extraneous protein in preparations of such infected tissue make it difficult to obtain pure preparations of soluble antigen.

Investigators in Mexico and Russia have demonstrated specific serologically active substances in the urine and blood of patients in the acute stage of typhus fever.[71, 72, 73] The precipitin test, the coated colloid particle agglutination reaction and the complement fixation technic have been used to detect the antigen, or antigens, which appear in the body fluids These studies show promise of providing a diagnostic method applicable early in the disease, but additional work along these lines is indicated

VI. SUMMARY OF THE SEROLOGIC REACTIONS IN RICKETTSIAL DISEASES

A summary of the *in vitro* and *in vivo* serologic reactions in the rickettsial diseases is given in Table 2 (pp. 326–27) Those technics which are of considerable diagnostic importance in each disease are listed in italics. Furthermore, this system of emphasis has been employed to indicate the more useful sources of antigen and antisera.

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